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# Regulation of macrophage subsets in homeostatic and inflammatory mucosal environments

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University of Plymouth

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# **Regulation of macrophage subsets in homeostatic and inflammatory mucosal environments**

**By**

**Khalid BinFahad B Alshaghdali**

A thesis submitted to the University of Plymouth

In partial fulfilment for the degree of

**Doctor of Philosophy**

School of Biomedical and Healthcare Sciences

Plymouth University Peninsula Schools of Medicine and  
Dentistry

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of Saudi Arabia

2018



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# Abstract

The interaction between epithelial cells and macrophages is integral to mucosal immune fate: determining the decision between tolerance and immune activation/inflammation. Endotoxin tolerisation (ET) is a circumstance where cells go through a hypo-responsive state, unable to respond to further endotoxin-LPS challenge. Mucosal macrophages (MΦs) have a dual functionality that determines tolerance to commensal organisms or immune response to entropathogens such as *E. coli*. In the case of mucosal inflammatory pathologies, such as Crohn's disease, this state of tolerance is broken, resulting in destruction of gut mucosal tissue where the macrophage phenotype has been altered from a regulatory M2-like subset phenotype to an inflammatory M1-like subset phenotype, responding to both pathogenic and commensal bacteria. Chronic inflammation by bacterial pathogen related molecular patterns (PAMPs), such as LPS, is well established to induce tolerisation. The aims of this project were firstly, to characterise the control of macrophage differentiation in a mucosal setting by investigating the immunomodulatory effects of PAMPs, such as LPS in presence or absence of TNFα and to investigate ET mechanisms associated with MΦ subsets responding to the entropathogen *E. coli* K12-LPS. Secondly, to investigate the effect of epithelial cells on macrophage subsets behaviour upon inflammation and ET. M1- and M2-like MΦs were generated *in vitro* from the THP-1 monocyte cell line by differentiation with PMA and vitamin D<sub>3</sub>, respectively, whereas differentiated epithelial cells (Caco-2) were obtained by long term culturing for 21 days. A transwell co-culture system of Caco2 cells and MΦ subsets was developed to mimic the cell-to-cell cross-talk between epithelial cells and immune cells. Mono- and co-culture models were pre-treated with either LPS, TNFα or IL-1β prior to stimulation by PAMPs. TNFα, IL-1β, IL-18, IL-6 and IL-10 were qualified by ELISA. Cytokines, PRRs and endogenous negative regulatory molecules were detected by RT-PCR and WB and epithelial barrier function was measured by trans epithelial electrical resistance (TEER). ET induced by K12-LPS failed to demonstrate a differential subset-specific response in MΦ mono-culture system whereas, LPS differentially suppress LPS induced cytokine expression in MΦ co-culture system. Tolerised M1- and M2-like MΦs exhibited a significant reduction in expression and secretion of pro-inflammatory cytokines and comparable levels of anti-inflammatory cytokine, IL-10. The suppression of pro-inflammatory cytokine in these MΦs appeared to be linked to the differential TLR4 expression and up-regulation of negative regulators, such as IRAK-M and Tollip. In addition, MΦ subsets differentially responded to inflammation induced by pro-inflammatory cytokines, TNFα and IL-1β in mono- and co-culture models. In conclusion, tolerisation induced in MΦs is presented by the suppression of pro-inflammatory cytokine, which is associated with corresponding up-regulation of IL-10, TLR4 receptor and the negative regulators, in a subset-independent manner. In the case of cross-talk between epithelial cells and macrophages however, a differential sensitivities to ET was displayed. These findings allow more understanding of MΦ subsets functions and ET mechanisms, which may be beneficial for the development of *in-vitro* models of MΦ subsets and therapeutics targeting Crohn's diseases.

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# Author Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

Work submitted for this research degree at the Plymouth University has not formed part of any other degree either at Plymouth University or at another establishment.

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Signed:

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Date: 24.07.2018

# List of Publications

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## Conferences

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**Al-Shaghdali, K.**, Hayward, C., Beal, J. and Foey, A. (2014): Effect of endotoxin tolerance induced by E. coli-LPS on cytokine production of macrophage subsets. The British society for Immunology annual congress 1-4 December, Brighton, UK.

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# Abbreviations

<b>Abbreviation</b>	<i>meaning</i>
<b>ANOVA</b>	Analysis of variance
<b>APCs</b>	Antigen presenting cells
<b>ATP</b>	Adenosine triphosphate
<b>bp</b>	Base pair
<b>BSA</b>	Bovine Serum Albumin
<b>CD</b>	Cluster of Differentiation
<b>CD</b>	Crohn's disease
<b>cDNA</b>	Complementary Deoxyribonucleic Acid
<b>CO<sub>2</sub></b>	Carbon Dioxide
<b>DAMP</b>	Damage Associated Molecular Patterns
<b>DCs</b>	Dendritic Cells
<b>DMSO</b>	Dimethyl sulfoxide
<b>DNA</b>	Deoxyribonucleic Acid
<b>dNTP</b>	Deoxynucleotide Triphosphate
<b>DPBS</b>	Dulbecco's PBS
<b>DSS</b>	Dextran Sulphate Sodium
<b>DW</b>	Distilled Water
<b>ED<sub>50</sub></b>	50% effective dose
<b>EDTA</b>	Ethylene-Diamine Tetra-Acetic Acid
<b>ELISA</b>	Enzyme Linked Immune-Absorbent Assay
<b>ET</b>	Endotoxin tolerance
<b>FAE</b>	follicle - associated epithelium
<b>FCS</b>	Fetal Calf Serum
<b>Flag</b>	Flagellin
<b>GALT</b>	Gut Associated Lymphoid Tissue
<b>GIT</b>	Gastrointestinal Tract
<b>GM-CSF</b>	Granulocyte Macrophage Colony-Stimulating Factor

<b>h</b>	hour
<b>hBD-2</b>	human Beta Defensin -2
<b>HRP</b>	Horseradish Peroxidase
<b>IBD</b>	Inflammatory Bowel Disease
<b>ICT</b>	inhibitory control test
<b>IECs</b>	Intestinal Epithelial Cells
<b>IFN-<math>\gamma</math></b>	Interferon Gamma
<b>IKK</b>	Inhibitor of $\kappa$ B Kinase
<b>IL</b>	Interleukin
<b>iNOS</b>	Inducible nitric oxide synthase
<b>IRAK</b>	IL-1 Receptor Associated Kinase
<b>IRF</b>	- IFN-Regulatory Factor
<b>I<math>\kappa</math>B</b>	Inhibitor of $\kappa$ B
<b>KDa</b>	Kilo Dalton
<b>LBP</b>	LPS Binding Protein
<b>LPS</b>	lipopolysaccharide
<b>LTA</b>	Lipoteichoic acid
<b>M</b>	Molar
<b>M cells</b>	micro fold cells
<b>M<math>\Phi</math></b>	Macrophages
<b>M1 subset</b>	Macrophage (Pro-inflammatory M $\Phi$ )
<b>M2 subset</b>	Macrophage (Anti-inflammatory M $\Phi$ )
<b>MAPK</b>	Mitogen-Activated Protein Kinases
<b>MCP-1</b>	Macrophage Chemoattractant Protein-1
<b>M-CSF</b>	Macrophage Colony Stimulating Factor
<b>MDP</b>	Muramyl Dipeptide
<b><math>\mu</math>g/ml</b>	Microgram per millilitre
<b>mg</b>	milligram
<b>MHC</b>	Major Histocompatibility Complex
<b>min</b>	minute
<b>MyD88</b>	Myeloid Differentiation Factor 88
<b>NCBI</b>	National Centre of Biotechnology Institute

<b>NIBSC</b>	National Institute for Biological Standards and Control
<b>NF-<math>\kappa</math>B</b>	Nuclear Factor kappa B
<b>ng</b>	nanogram
<b>NODs</b>	Nucleotide Oligomerisation Domains
<b>NOS</b>	Nitric oxide synthase
<b>OD</b>	Optical Density
<b>PAMPs</b>	Pathogen Associated Molecular Patterns
<b>PBS</b>	Phosphate Buffered Saline
<b>PCR</b>	Polymerase Chain Reaction
<b>PGN</b>	Peptidoglycan
<b>PI3K</b>	Phosphatidylinositide 3-kinases
<b>PMA</b>	Phorbol-12-Myristate Acetate
<b>PPs</b>	Peyers' Patches
<b>PRRs</b>	Pattern Recognition Receptors
<b>R10</b>	RPMI supplemented with 10% v/v foetal calf serum (FCS)
<b>RPMI</b>	Roswell Park Memorial Institute
<b>RT- PCR</b>	Reverse Transcriptase Polymerase Chain Reaction
<b>RT</b>	Room Temperature
<b>SDS-PAGE</b>	Sodium-Dodecyl Sulphate-Polyacrylamide Gel electrophoresis
<b>SE</b>	Standard Error
<b>SIGIRR</b>	Single Immunoglobulin Interleukin I Receptor Related
<b>siRNA</b>	Small interfering RNA
<b>SNP</b>	Single nucleotide polymorphism
<b>SOCS</b>	Suppressor of cytokine signaling
<b>STAT</b>	Signal Transducers and Activators of Transcription
<b>TAMs</b>	Tumor associated macrophages
<b>TEER</b>	Transepithelial Electrical Resistance
<b>TGF-<math>\beta</math></b>	Transforming Growth Factor Beta
<b>Th</b>	T helper cell
<b>TLR</b>	Toll-Like Receptor
<b>TNF<math>\alpha</math></b>	Tumour Necrosis Factor Alpha
<b>Tollip</b>	Toll Inhibitory Protein



<b>TRAF6</b>	TNF Receptor Associated Factor 6
<b>UC</b>	Ulcerative Colitis
<b>UV</b>	Ultra Violet
<b>VD<sub>3</sub></b>	1,25-(OH) <sub>2</sub> -Vitamin D <sub>3</sub>
<b>v/v</b>	Volume by volume
<b>WB</b>	Western Blot
<b>w/v</b>	Weight by volume

# **Chapter 1: General Introduction and literature review**

## **1.1 Introduction**

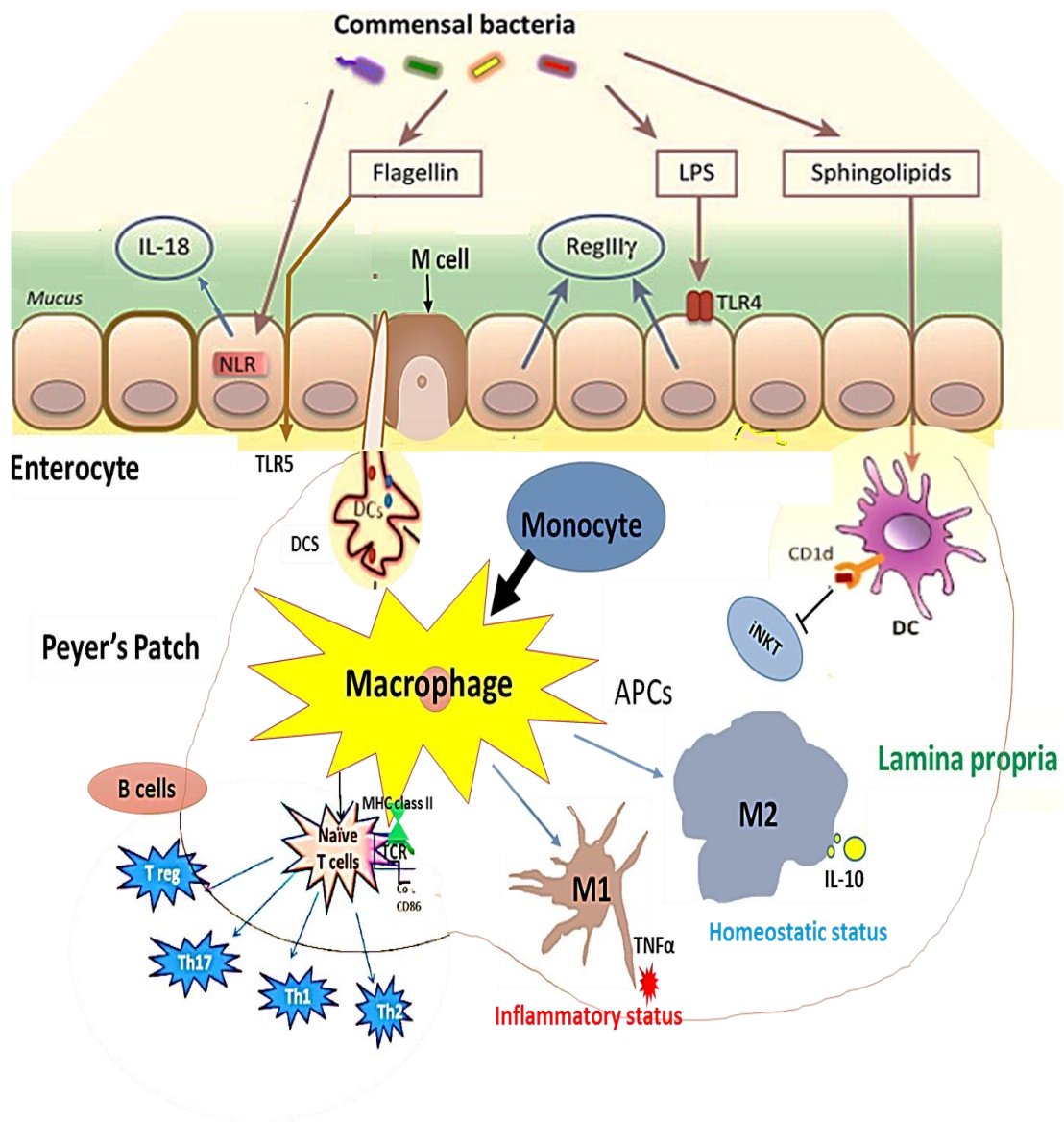
### **1.1.1 Innate immunity in inflammatory bowel disease**

The gastrointestinal mucosa is frequently exposed to bacterial and food antigens from varied resident microbial flora (Gill et al., 2006). The intestinal commensal flora is of advantage to the host via the metabolism of food elements that are not absorbed and the vitamins that are consequently produced such as Vitamin B12 and vitamins C. However, the gut lumen might also be the location where pathogenic microorganisms enter the body, causing the potential for tissue damage. Consequently, intestinal innate immunity is a sensitive system of ensuring a swift, appropriate immune reaction towards pathogenic bacteria, whilst simultaneously permitting food and beneficial bacterial antigens to enter the body. An effective epithelial boundary and an intricate, developed immune system in the gut enable this multi-dimensional objective to be achieved. If this delicate compromise is disturbed, then an aberrant inflammatory reaction might occur, resulting in chronic intestinal inflammation, for example inflammatory bowel disease (IBD). IBD is a chronic inflammatory condition of the gastrointestinal tract that incorporates Crohn's disease (CD) and Ulcerative Colitis (UC). Approximately 1 in 1000 individuals suffer from IBD within Europe, with enhanced levels of the disorder evident within westernised and industrialised nations (Loftus, 2004). Both UC and CD have been connected with an enhanced danger of intestinal cancers. The predominant symptoms include pain in the abdominal area, rectal bleeding and diarrhoea. However, the condition can also occur in extra-intestinal areas, such as the eyes, kidneys, skin and joints (Bernstein et al., 2001). Individuals suffering from IBD additionally have an enhanced danger of suffering from further chronic

immune conditions, including psoriasis, ankylosing spondylitis and primary sclerosing cholangitis, the condition of shrinking of the intra-and extrahepatic biliary tree minor to fibrosis and inflammation of the submucosa (Bernstein et al., 2005, Werlin et al., 1980). Weaknesses in epithelial barrier function, innate immune recognition and immune regulatory mechanisms may increase the aberrant expression of pathological inflammatory reactions in IBD (Geboes, 2001). It is commonly suggested that the pathogenesis of IBD is a consequence from the irregular immune response to enteric bacteria, leading to overproduction of pro-inflammatory cytokines such as TNF $\alpha$  and IL-1 $\beta$ , promoting tissue injury of the gut mucosa. Treatments for individuals suffering from IBD include attention being paid to their lifestyle and suitable changes introduced, as well as medical and surgical processes. The critical implicated of TNF $\alpha$  in IBD pathophysiology was emphasised by the successful use of anti-TNF treatments for patients who have CD (Targan et al., 1997). The medical stage includes corticosteroids, immunosuppressant agents and biologic treatments, including varying anti-tumour necrosis factor elements (TNF $\alpha$  compounds) (Baumgart and Sandborn, 2007). Yet none of these processes can provide a full cure and each have varying side effects. The origins of IBD are still not clear; although it is believed that genetically susceptible people are liable to suffer from it due to an unusual immune reaction towards the microorganisms of the intestinal flora. There is evidence to indicate that the dysregulated innate and adaptive immune pathways result in an abnormal intestinal inflammatory reaction in individuals suffering from IBD. The majority of research undertaken within the last three decades has placed its emphasis on the function of aberrant adaptive immune reactions in the origins of IBD. Whilst CD has frequently been perceived as being instigated by a Th1 reaction, UC has tended to be connected to an unconventional Th2 reaction (Fuss et al., 1996). As well as typical Th1 and Th2

reactions, a function for Th17 cells has also become evident (Geremia and Jewell, 2012). Progress resulting from genome-wide association studies (GWAS) and immunological studies has latterly led the emphasis of IBD's origins towards mucosal innate immune reactions, including innate microbial sensing, epithelial barrier integrity, unfolded protein reaction and autophagy (Geremia et al., 2014).

The innate immune reactions indicate our initial defensive barriers. In contrast to the adaptive reaction, it is not a specific response and does not involve long-term immunity in the form of memory. The immune cells within the innate system, including Dendritic cells (DCs) and macrophages (MΦs), as well as intestinal epithelial myofibroblasts, are able to detect the intestinal microbiota and react to pathogen associated molecular patterns (PAMPs) in a typical way. This process enables a swift and efficient inflammatory reaction against the microbial process to occur. Additionally, DCs and MΦs are antigen presenting cells, which initiate T cell commencement and lead to the adaptive immune reaction.



**Figure 1.1-1: The role of MΦs and innate cells in primary responses in the gut associated lymphoid tissue (GALT).**

GALT contains Peyer's Patches (PPs), which consist of follicles consisting of Micro-fold cells (M cells), T, B cells, Dendritic cells (DCs), monocytes, and macrophages. T cells differentiated into Th1, Th2, Th17, and Tregs. Macrophages differentiated into either M1-pro-inflammatory ( $\text{TNF}\alpha^{\text{hi}}$ ) or M2-anti-inflammatory ( $\text{IL-10}^{\text{hi}}$ ) subsets. Antigens cross via M cells through endocytosis or by DCs over, phagocytosis. Antigen processing and presentation is prepared by antigen presenting cells (APCs) such as macrophages and DCs. APCs present antigens to T cells via MHC class II, recognised by T cells by the T cell receptor (TCR). In response to antigen presentation and depending on the type of stimuli, T cells either show immune activation or mucosal tolerance. Recognition of commensal-derived PAMPs, such as LPS, TLR4 agonist, by the intestinal epithelial cells (IEC) induces secretion of the antimicrobial peptide. Microbiota-derived signals induce IL-18 production from the IEC through activation of NOD-like family, receptors (NLRs). Microbiota-derived sphingolipids presented on CD1d by DC inhibit colonic invariant natural killer T cells (iNKT) development. This figure is adapted from information presented in (Kabat et al., 2014).

### 1.1.2 Intestinal epithelial barrier function

The initial physical restriction that intestinal bacteria and food antigens must face is in the form of the mucous layer that protects the intestinal epithelium. Mucous is divided into an inner firm layer and a looser outer layer, and results from gel-generating mucins being polymerised by goblet cells and expand in the lumen because of their ability to bind water. The consequential mucin net is generally disinfected, whilst the outer mucous sections seems to be more porous and is occupied by commensal bacteria that find essential nutrients in the mucin glycans. The importance of mucous is in limiting bacterial advancement and intestinal inflammation, which has been emphasised by research conducted into MUC2<sup>-/-</sup> mice that contract colitis and are at increased danger of suffering from colorectal cancer (Van der Sluis et al., 2006, Velcich et al., 2002). Whilst there is not a mutation within the human MUC2 gene that has been connected with disturbing the purpose of the mucosal barrier, limited MUC1 mRNA has been detected within the inflamed ileum of CD individuals MUC3,4 and MUC5B within the uninflamed ileum when a contrast is made with the control (Buisine et al., 1999). Gut associated lymphoid tissue (GALT) comprises enterocytes and specialised epithelial cells (

Figure 1.1-1), including goblet cells and Paneth cells. Paneth cells are only evident within the foundation of the crypts within the small intestine. Tight junctions, adherens junctions and desmosomes are used to preserve the integrity of the epithelial barrier. Defective epithelial barrier and enhanced intestinal permeability have frequently been detected in individuals suffering from CD and UC (Salim and Söderholm, 2011). Yet it was still not obvious whether these changes caused chronic inflammation or resulted from it. GWAS data available recently indicates that epithelial

barrier difficulties indicate a primary pathogenetic mechanism. Recently, UC has been connected with susceptibility single nucleotide polymorphisms in HNF4A. This is a transcription factor that controls the compilation of CDH1, the apical junction complex (Barrett et al., 2009). This encodes for E-cadherin, which is a prime element of adherents junction and a significant element in meditating the process of the epithelial intercellular communication, LAMB1, a laminin which occurs within the basal membrane of the intestinal epithelium, and GNA12, encoding for a GTPase. This occurs at tight junction formation via a combination of ZO-1 and Src (Barrett et al., 2009, Anderson et al., 2011). As well as creating a physical defence against infection from bacteria, epithelial cells are able to secrete a variety of bactericidal agents such as defensins ( $\alpha$ -defensins result from Paneth cells whilst the majority of epithelial cells produce  $\beta$ -defensins). These agents are either generated constitutively, or through being induced via the identification of bacterial elements by pattern recognition receptors (PRRs) within epithelial cells (Uehara et al., 2007). The diminished induction of the  $\beta$ -defensins HBD2, which targeting Gram-negative bacteria (*E. coli*) (Harder et al., 1997), HBD3 and HBD4 has been identified within the inflamed colon of individuals with CD in contrast to UC (Wehkamp et al., 2003). Furthermore, individuals with levels of ileal CD had reduced levels of the Paneth cell-derived  $\alpha$ -defensins (HD5 and HD6) (Wehkamp et al., 2005). The diminished expression of HD5 and HD6 is particularly evident in patients containing the NOD2 frameshift susceptibility variant, indicating that an antimicrobial peptide hindrance might explain at least one of the elements accountable for the dangers connected with NOD2 (Wehkamp et al., 2004).



### **1.1.3 Intestinal Macrophages and Macrophage subsets**

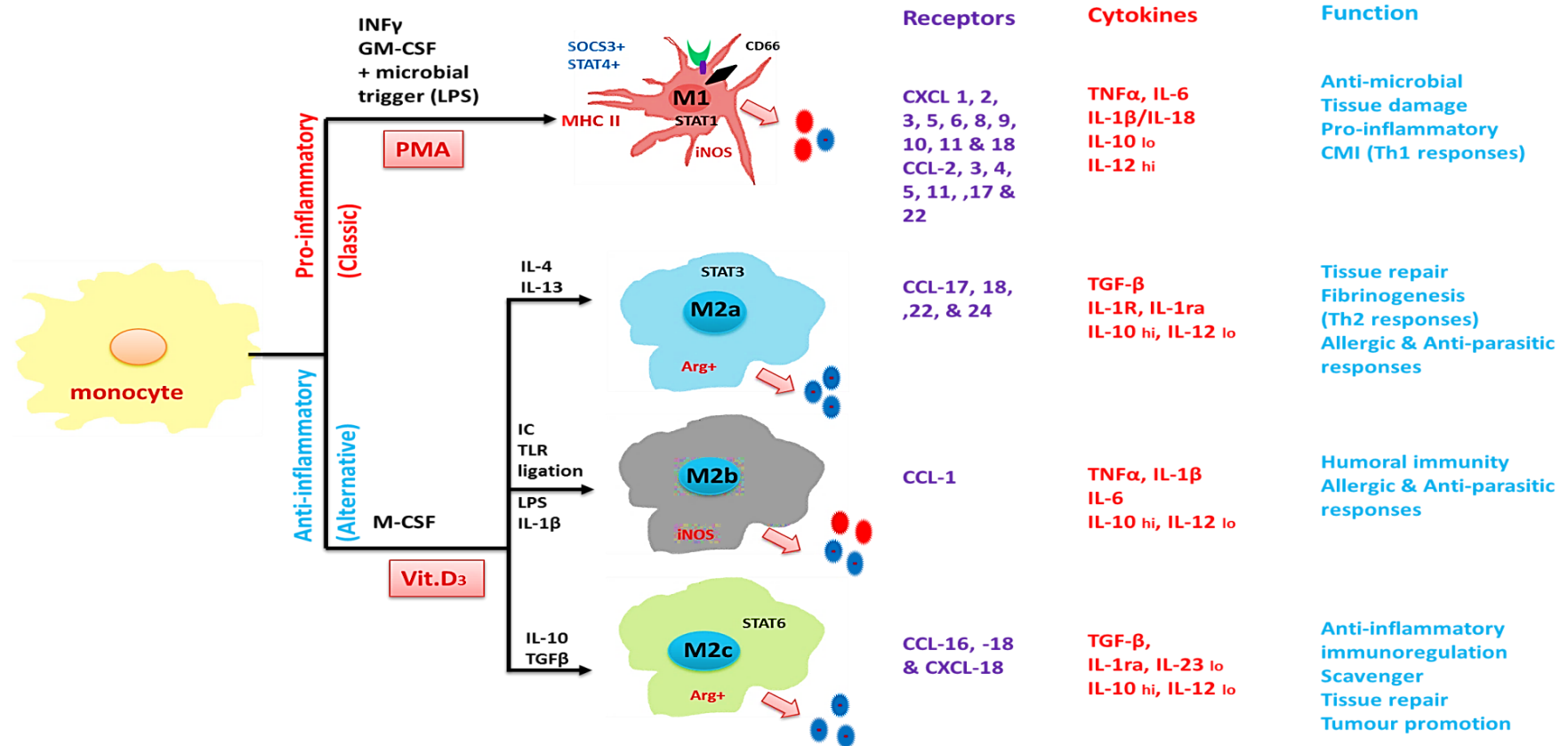
Macrophages refer to heterogeneous populations of antigen presenting cells (APCs). These are present in most tissues and detect and eradicate pathogens and toxic molecules that are invading. MΦs were initially described by Elie Metchnikoff in 1882; they might be regarded as mononuclear phagocytic immune cells are an important element in the early and changing responses of the immune system (Cavaillon, 2011). MΦs predominantly stem from circulating monocyte precursors, once they move from the blood vessels into the endothelium, they eventually reach the tissue and divide into macrophages when exposed to infection or inflammatory stimuli (Hume, 2006). Tissue MΦs act as a form of specialised-tissue resident macrophage that are present across the body, and involved in homeostatic roles, including Kupfer cells in the liver, alveolar MΦs in the lung, microglial MΦs in the brain and osteoclasts in bones. As macrophages are present in virtually all organs and tissues within the body, it is to be expected that they are involved in preventing and resisting a range of chronic conditions. One example of this is cancer, whereby MΦs are recruited to the tumour microenvironment. They are referred to in this instance as tumour associated macrophages (TAMs). TAMs have a central influence in acceleration of the progression of tumours and malignancy, such as advancing angiogenesis (Bingle et al., 2006, Mantovani et al., 2006). As such, there is a link between TAMs and poor prognosis within virtually 80% of cancer situations in humans (Bingle et al., 2002). Other cases of them affecting autoimmune conditions are Crohn's disease, an infectious inflammatory autoimmune condition, connected to the pro-inflammatory cytokines, IL (interleukin)-12 and IFN (interferons)  $\gamma$ . This is associated which is linked to the pro-inflammatory situation (Bouma and Strober, 2003, Foey, 2015). MΦs are also associated with the advancement of acute and chronic rheumatoid arthritis (RA), a sterile inflammatory

autoimmune condition, in which MΦs become active within various inflamed synovial membranes (Mulherin et al., 1996, Kinne et al., 2000). As such, pro-inflammatory revealed to have a considerable influence within the pathology of RA via TNFα controlled ROS generation (Miesel et al., 1996). As such, pharmacological modulation of the MΦ immune role may be effective in attempting to resolve inflammation linked to disease, such as rheumatoid arthritis. As such, the range of specialised macrophages indicate the heterogeneity of the process (Gordon and Taylor, 2005). Anti-TNFα therapy has been used for the treatment of gastrointestinal inflammation. MΦs have a variety of beneficial functions, including eliminating microbial, reacting against tumours, and healing wounds (Vega and Corbí, 2006). The range of these functions is indicated by the variety of specific distinct macrophage subpopulations, which is thought to be influenced by a range of aspects such as microenvironment, differentiation and heterogeneity (Foey, 2015).

Within the colon and small intestinal mucosa, MΦs are located slightly below the epithelium within the lamina propria (LP). Within the small intestine, which incorporates somewhat reduced amounts of mucosal MΦs in contrast to the colon (Lee et al., 1985), they are also evident within the sub-epithelial dome area of Peyer's Patches (PP) (

Figure 1.1-1). Due to their positioning, mucosal MΦs are suitably located to interact directly with the bacteria in the lumen and to identify any microbes or microbial processes that might cross the epithelial monolayer. This might happen in varying ways. For example, MΦs might act in a similar way to mucosal DC, which have been reckoned to convey processes amongst intestinal epithelial cells (IEC) and into the lumen when responding to indications that epithelial cells have detected bacteria

(Niess et al., 2005, Chieppa et al., 2006). Other potentials include antigen acquisition by breaches of the intestinal barrier, or indirectly through uptake of dying epithelial cells that have innate antigen (Mowat, 2003). MΦs might obtain bacteria via a non-direct route, after the transfer process from IEC. It is possible to identify pro-inflammatory mediators that the IEC releases when detecting products like lipopolysaccharide (LPS) (Hornef et al., 2003). Typical MΦs identify microbes as part of the PRRs process, which identify structured motifs on prokaryotic and lower eukaryotic organisms. The most effective of these are toll-like receptors (TLRs), which detect pathogen-associated molecular patterns (PAMPs), like bacterial LPS, lipoprotein, lipoteichoic acid, flagellin and unmethylated CpG that incorporate DNA. As a rule, macrophages can be divided into two specific forms: M1 (classical) and M2 (alternative) macrophages. These are a paradigm of Th1/Th2 (T helper 1/2) polarization (Mills et al., 2000). When considering macrophage heterogeneity, it is unsurprising that macrophages reflect plasticity and fluidity due to their heterogeneity. As such, their polarised subsets exhibit a range of response when reacting to environmental stimuli (Mantovani et al., 2004). Furthermore, more MΦ subsets have been described, particularly related with study of inflammatory pathologies such as atherosclerosis include include M4, Mox, HA-mac, M(Hb) and Mhem (Colin et al., 2014, De Paoli et al., 2014) and reviewed in (Foey, 2015). However, with the absence of defined transcription factors that would normally show lineage commitment, it could not be agreed that these are distinct subset although they have been described.



**Figure 1.1-2: Functional phenotypes of macrophage subsets.**

Monocytes are differentiated into different macrophage effector phenotypes by a variety of signs. GM-CSF,  $\text{INF}\gamma$ , and LPS trigger the pro-inflammatory MΦs, M1, while M-CSF, IL-4/IL-13, immune complexes (IC) +TLR ligation, IL-10, TGF- $\beta$  and glucocorticoids trigger the anti-inflammatory MΦs, M2. Macrophage functions are formed as results of several factors include inducible nitric oxide synthase (iNOS), arginase (Arg) expression, transcriptional factors (STAT-1, 3, 6), scavenger receptors (SR) such as mannose receptors MR, MHC II, co-stimulatory ligands (CD80, CD86) and cytokines (IL-12, IL-23, IL-10, TGF- $\beta$ ), chemokines (CC-and CXC) expression. This figure is adapted from information presented in (Foey, 2015, Gordon, 2003, Mantovani et al., 2004).

### **1.1.4 Macrophage polarisation**

Macrophages are part of a heterogeneous myeloid-monocytic lineage that can be polarised when responding to a range of environmental symptoms (Mantovani et al., 2004), such as cytokines, bacterial elements and chemokines. As such, classical activation M1s are induced by IFN- $\gamma$  separately or else when combined with classical stimuli, including TNF $\alpha$  or LPS. Furthermore, classical M1s can be induced when they are combined with the PKC activating DAG analogue (phorbol myristate acetate, PMA) (Kielian and Cohn, 1981, Green and Phillips, 1994). Granulocyte macrophage colony stimulation factor (GM-CSF) is a further element that can increase the variation of M1s within a concept of human monocytes (Finnin et al., 1999). However, M2s are induced by the Th2-derived cytokines, IL-4 with IL-13. Together, it is recognised that this results in M2-like mediated tumour expansion and advancement (Anderson and Mosser, 2002, Mantovani et al., 2002, Gordon, 2003).

M1s exhibit increased anti-microbial levels and secrete significant amounts of pro-inflammatory cytokines (TNF $\alpha$ , IL-1 $\beta$ , IL-18, IL-6 and IL-23), and express chemokines (CXCL 1, 2, 3, 5, 8, 10, CCL3, 4, 5, 11, 17 and 22; (Figure 1.1-2). Another attribute of M1s is the fact that they express inducible nitric oxide synthase (iNOS) which is incorporated in L-arginine catabolism, culminating with the generation of nitric oxide (NO) that is recognised as a significant mediator of macrophage cytotoxicity (Nathan and Hibbs, 1991). In fact, it has been indicated that iNOS expression correlates with the impact of antimicrobial activity upon NO (Granger et al., 1988). It is notable that the iNOS mRNA translation was restructured by arginase-mediated arginine depletion being induced (Lee et al., 2003). Furthermore, it is recognised that macrophages increase the production of ROS, including superoxide anions and hydroxyl radicals in to

response virus disease (Sindrilaru et al., 2011, Afonso et al., 2007). Toxic intermediates and ROS are regarded as significant elements of classical activation; M1s utilise them to enhance their processes of killing bacteria when initially responding to an invading pathogen. There is the argument that TLRs, especially TLR 2, 4 and 5, which have a direct correlation with NF- $\kappa$ B (nuclear factor-kappa B) activation and pro-inflammatory cytokine secretion, are a distinct element within LPS-induced M1s (Bosisio et al., 2002, Van Genderachter et al., 2006, Foey, 2015). However, IL-13 and IL-4 mediation of M2 activation was indicated to oppose these receptors (Bosisio et al., 2002, Gordon, 2003). Each of the family receptors (TLRs/ NOD-like receptor (NLRs)) has a pivotal function within activating immune cells, including neutrophils and macrophages (Fukata et al., 2009). M1 activation also incorporates the upregulation of MHC II (HLA-DR, surface molecule), co-stimulatory B-7.2 (CD86; cluster distinctiveness 86) and IL-12 improving the capacity of activating Th1-mediated reactions to resident pathogens and to improve high protection (Fukata et al., 2009, Hölscher et al., 2001, Mosser, 2003). This upregulation in expression, coupled with IL-23, as generated by M1s, sustains the distinctiveness and purpose of Th17 cells (Stockinger and Veldhoen, 2007). Overall, by the expression matrix metalloproteinases, M1s can be identified (MMP-1,-2,-7,-9 and -12) under inflammatory conditions (Gibbs et al., 1999, Van Genderachter et al., 2006). M1 polarisation can additionally relate to transcription elements such as STAT1, utilised by IFN $\gamma$  and GM-CSF, and nuclear factor (NF)- $\kappa$ B which relate to pro-inflammatory cytokine expression being induced (Liu et al., 2008). Activated macrophages partake in the role of eliminating micro pathogens, tissue damage and pro-inflammatory reactions through deriving Th1 response (Mills et al., 1992, Mills et al., 2000). In contrast, M2s (alternative activation) relate to immune control, tissue repair capacity, and advancing tumours and angiogenesis by suppression of Th1

inflammatory responses and stimulation of the Th2-mediated anti-inflammatory response that is consequently stimulated (Anderson and Mosser, 2002, Lopez-Castejón et al., 2011, Takeuchi and Akira, 2011). It has been proven that M2s contain a notable activation feature that incorporates extensive expression (Mantovani et al., 2002, Gordon, 2003) of mannose receptor (MR, CD206) which is a notable element within the anti-inflammatory phenotype (Figure 1.1-2). When activated alongside IL-4, additional important markers (arginase I, Arg+ I) have been proven to be upregulated within the M2s. This leads to into ornithine and urea. This is subsequently catabolised into polyamines and proline, substances essential for cellular proliferation and tissue repair (Gordon, 2003, Gordon and Taylor, 2005, Classen et al., 2009, Ochoa et al., 2001). Arginase has a role in tumour invasion as it generates myeloid immune suppressor cells within individuals suffering from cancer (Zea et al., 2005). These enable immune inhibitory mechanisms, significantly contributing to the prevention of disease (Highfill et al., 2010). Due to the cytokine profile being a form of macrophage polarization, M1s have the attributes of IL-12 high and IL-10 low. In contrast, M2 macrophages are responsible for diminishing pro-inflammatory cytokines and as such their attributes are IL-10 high and IL-12 low (Mantovani et al., 2004). In a manner similar to the cases of IL-4 and IL-13, alternative activation stems from being exposed to IL-10, M-CSF, glucocorticoids or alternative steroid (vitamin D<sub>3</sub>) signals (Goerdts and Orfanos, 1999, Mantovani et al., 2004). Lastly, alternative M2s can be categorised into three groups. M2a can be induced when they are exposed to IL-4 or IL-13 and secrete TGF- $\beta$  (tumour advancement elements-  $\beta$ ); IL-1R and IL-1ra, while expressing CCL-17, 18, 22 and 24 (Figure 1.1-2). On the other hand, M2b macrophages can be induced via immune complexes (IC) and TLRs agonists or IL-1 $\beta$  receptors, secrete cytokines including TNF $\alpha$ , IL-8 and IL-1 $\beta$  and express CCL-1, which recruited M2b. (Figure 1.1-2).

Immunosuppressive M2c can be induced via IL-10 and TGF- $\beta$  or glucocorticoids, secrete TGF- $\beta$  and IL-23<sup>lo</sup> and express CCL-16 and 18, alongside CXCL-18. Furthermore, M2b and M2c are regarded as being regulatory macrophages (Gordon, 2003, Martinez et al., 2009).

### **1.1.5 Innate immune recognition**

The primary function of PRRs involves providing a defence towards pathogens. In contrast, gut epithelial PRR recognition of conventional commensal PAMPs are transitioned into signals for expressing anti-microbial peptide (hBD-2), improving barriers (ZO-1 being reallocated), and the proliferation of epithelial cells (Cario and Podolsky, 2005). As such, cell signalling being weakened leads to polymorphism in TLR and NLR genes that is implicated in the spread of numerous conditions (Franchimont et al., 2004, Hedl et al., 2007). PRR signalling cascades lead to NF- $\kappa$ B activation with gene transcription and the generation of pro-inflammatory mediators lead to a suitable innate reactions towards pathogens. TLRs detect PAMPs being located extracellularly or within intracellular vacuoles, for instance lysosomes or endosomes. Stimulation of TLR by their relevant ligands, triggers intracellular cascades that are classically myeloid differentiation primary response gene 88 (MyD88)-dependent and MyD88-independent pathways which incorporate IL1 receptor associated kinase (IRAK) and TNF receptor associated factor 6 (TRAF6) (Akira et al., 2006). The TLR family contains the human homologues of the *Drosophila* Toll protein. The extracellular domain of Toll receptors, meanwhile, comprises a leucine-rich repeat (LRR) whilst the cytoplasmic domain presents homology alongside the interleukin-1 receptor (IL-1R). This is referred as the Toll/IL-R (TIR) domain. TLRs do not only detect and react to pathogen associated



molecular trends, but also join with non-pathogenic antigens including heat shock proteins(O'Neill and Bowie, 2007).

The localisation of TLRs and NLRs is a vital function in regulating inflammation and preserving the typical gut mucosa. The expression of TLRs differs within cell types and cellular localisation. TLRs are expressed within a number of myeloid cells, including macrophages and DCs, and on non-myeloid cells, including epithelial cells and fibroblast. This expression can be extracellular - for example (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11), intracellular (TLR3, TLR7, TLR8, TLR9) - or both (TLR4, TLR9). The immune reaction materialised once PAMPs are detected via PRRs (when the extracellular LRR domain interacts with microbial PAMPs), with the recruitment of cytoplasmic adaptor (MyD88) then occurring, producing the activation of NF- $\kappa$ B (Wesche et al., 1997) and reviewed in (Takeda et al., 2003).

TLR4 was the first human TLR to be detected. This reacts to LPS from Gram-negative bacteria. The lipid A element is required within the LPS molecule to be detected by TLR4. This process results in the generation of an array of immunostimulatory cytokines and chemokines mediated by signaling intermediate MAPKs (mitogen-activated protein kinases) and the transcriptional factors AP-1, NF- $\kappa$ B and IRF5 pathways. In terms of signalling, TLR4 is reliant on CD14 and MD-2 being present so as to produce a complex (Shimazu et al., 1999). Similar to TLR1, 2 and 3, it was identified that TLR4 is expressed by monocytes, as well as by polymorphonuclear (PMN) cells and DCs, at reduced levels in B cells, on fibroblasts, astrocytes, keratinocytes, myocytes, endothelial cells and epithelial cells (Hornung et al., 2002, Song et al., 2002, Frantz et al., 1999, Bsibsi et al., 2002). A number of endogenous ligands for TLR4 have been defined but, since many of these investigates were shown with recombinant proteins

from *E. coli*, in reflection it is likely that some of the results stem from the usage of contamination with endotoxin (LPS). For example, heat shock protein Hsp70 has been explained as being endogenous TLR4 ligand by a variety of groups (Asea et al., 2002, Dybdahl et al., 2002). However, subsequent research was unable to verify these findings and indicated that tested, LPS-free Hsp70 had no TLR4 dependent effects (Bausinger et al., 2002, Gao and Tsan, 2003b, Gao and Tsan, 2003a, Wallin et al., 2002). Additional endogenous ligands have subsequently been expressed for TLR4. Yet it was not always researched as to if their activating properties could be due to being tainted by LPS. Fibrinogen, fibronectin, heparan sulfate, and hyaluronan, extra-cellular matrix proteins like, were also presented to stimulate TLR4 as endogenous ligands (Smiley et al., 2001, Okamura et al., 2001, Johnson et al., 2002).

IECs are structurally polarised where they are apically facing the intestinal lumen and basolateral facing the basement membrane and the lamina propria. It is suggested that TLR signalling in intestine could be regulated according to their location as apical or basolateral expression (Abreu, 2010). TLRs polarised expression by IECs at both apical and basolateral surface might lead to the discrimination between the pathogen and non-pathogen microbial signals (Peterson and Artis, 2014). Differential sensitivity of IECs to apical in contrast to basolateral stimulation has been demonstrated in in vitro and in vivo models with several TLR ligands (Gewirtz et al., 2001). For instance, basolateral contact of IECs to TLR9 ligands consequences in translocation of NF- $\kappa$ B and canonical activation whereas, apical contact leads to clear inhibitory result over the maintenance of I $\kappa$ B. Therefore the apical signals could induce tolerance to following TLR stimulation, represent a different cross tolerance alteration of microbial recognition pathways for the a differential responses to PAMPs signals according to

structural location (Lee et al., 2006). Another study assumed that TLRs expression might be selectively regulated in association with IBD and these modifications may be particular to the IBD type either CD or UC. Consequently, TLR3 and TLR4 are differentially regulated in the intestinal epithelium of IBD patients (Cario et al., 2000). Additionally, in the human foetus, it was presented that TLR2 and TLR4 were expressed on the basolateral side of ileal basement enterocytes (Fusunyan et al., 2001). The expression of TLR2 and TLR4 is low in healthy colon whereas, TLR4 is highly expressed on colon apical side in Crohn's disease patients (Cario and Podolsky, 2000). Other TLRs also seem to be polarised in IECs. Depending on the polarised cell line, LPS apical stimulation of Caco-2 cells results in NF- $\kappa$ B activation and IL-8 production (Lenoir et al., 2008). The different reactions following to apical or basolateral TLR activating are consistent with the suggestion that inflammatory and possibly negative TLR reactions to PAMPs must only happen when there has been a break in the epithelial barrier upon inflammation. In this manner, aggressive bacteria, mostly pathogenic bacteria, which can breach the epithelial barrier, provoke pro-inflammatory reactions; however, bacteria that cannot cross the barrier, generally non-pathogenic bacteria, stay on the apical side eliciting a homeostatic atmosphere and anti-inflammatory response (Abreu, 2010).

LPS, an important inducer of the inflammatory reaction to Gram-negative bacteria, is a large molecule comprising of O antigen, core, and lipid A. It incorporates two forms smooth and rough (Moreno et al., 1979). Furthermore, it functions as an endotoxin, and instigates a notable immune reaction via the Lipid A portion (Raetz and Whitfield, 2002). Indeed, the removal of O antigen leads to rough LPS, resulting in the bacterial cell membrane becoming more permeable and thus it is not so difficult for AMPs to

damage it. LPS is predominantly detected via TLR4 (Beutler, 2000), especially joining the lipid A section of rough LPS when CD14 and MD-2 were present (Taniguchi et al., 2009). In contrast, smooth LPS binds LPS binding protein (LBP) within the form of CD14 and MD-2 (Wright et al., 1990). It has been identified that MD-2 protein results in a complex with TLR4. This is crucial for surface expression and LPS responsiveness. As such, MD-2 plays a role in mediating the signal between TLR4 and LPS (Shimazu et al., 1999).

CD14, a co-factor for LPS signalling, described as a glycosylphosphatidylinositol (GPI)-linked membrane protein. This does not have the capacity to implement a trans-membrane signal, as it does not contain the cytoplasmic domain. As such, in order to mediate signalling, it is required to combine with other proteins. There are varying purposes to CD14 expression. Jean et al. (2001) asserted that LPS is only precisely cross-linked TLR4 and MD-2 when it interacts with CD14 (da Silva Correia et al., 2001). As well as its function in LPS detection, CD14 also notes bacterial PAMPs; peptidoglycan (PGN), mycobacterial lipoarabinomannan and streptococcal cell wall polysaccharides (Pugin et al., 1994, Soell et al., 1995, Weidemann et al., 1997, Wright et al., 1990). Therefore, it has the capacity to detect and join elements of both Gram-negative and Gram-positive bacteria and to serve as a co-receptor for TLR4 and TLR2. Additionally, CD14 can cause natural reactions to non-self-molecules that have the potential to infect, whilst also combining with apoptotic cells, to act as a scavenger receptor in macrophage cells (Devitt et al., 1998, Pradhan et al., 1997). CD14 has two types, soluble and membrane bound protein, which are expressed by a wide array of cells including monocytes/macrophages and epithelial cells (da Silva Correia et al., 2001). Recent research has confirmed that endogenous molecules or damage-

associated molecular patterns (DAMPs) exuded by damaged tissues have the capacity to activate CD14, the co- receptor of TLR4 and TLR2 (Yu et al., 2012).

TLR2 detects microbial PAMPs for both Gram-negative and Gram-positive bacteria, such as bacterial lipopolypeptides, peptidoglycan (PGN), lipoteichoic acid (LTA), and zymosan originating from cell wall component, yeast. It has a role in detecting Gram-positive bacterial products (PGN) as well as CD14 (Von Meyenburg et al., 2004). TLR2 expression has a major purpose in preserving gut mucosa. Latterly, researchers have indicated that TLR2 being induced by epithelial cells, as part of a reaction to conventional non-pathogenic microbiota, plays a part in the process of inducing the regulatory cytokine, IL-10, by macrophages and Tregs resident in the lamina propria resulting i gut tolerance (Round et al., 2011). As well as this, Cario (2008), utilising findings from recent animal model and human research, suggested that the shortcomings of TLR2 signalling might result in inconsistencies with the commensal-dependent intestinal epithelial barrier defences, leading to mucosal injury. Research indicated that polymorphism of TLR2 is linked to extensive bacterial infections, including *Staphylococcus* and *Mycobacterium* infection (Cario, 2008). This indicates that the TLR2 gene might increase the restriction of the host response to a particular array of microbial pathogens (Lorenz et al., 2000, Texereau et al., 2005), resulting in the initiation of intestinal injuries (Candia et al., 2012).

TLR5 recognises flagellin, the predominant structural protein of the flagella on Gram-negative and Gram-positive bacteria (Oshima et al., 2010). TLR5 has a significant function in preserving gut mucosa by inducing anti-microbial peptides, especially hBD-2 (Schlee et al., 2007); as such, the absence of it is connected with gut injury (Carvalho et al., 2012).

TLR9 detects unmethylated CpG bacterial DNA (Minns et al., 2006). The expression of TLR9 by epithelial cells has a significant function in controlling innate immunity via its function in degranulation of antimicrobial peptides such as defensins by Paneth cells (Rumio et al., 2004). Conventionally, the expression of TLR9 occurs basolaterally on epithelial cells; on the other hand, it is additionally on the apical cell surface in reaction to pathogenic microbial infection (Ewaschuk et al., 2007). Apical expression of TLR9 permits the cells to detect pathogenic bacterial DNA; basolateral expression, in contrast, detects commensal non-pathogenic bacteria instead (Lee et al., 2006).

TLR3 detects double stranded viral RNA (Alexopoulou et al., 2001), expressed by mature colonic epithelium towards viral infection. After the initiation of the TLR3 expression, there follows the intracellular activation downstream of the cells that are unrelated to the myeloid differentiation factor 88 (MyD88) pathway. This contrasts from other TLR cell initiation pathways (O'Neill, 2008, Yamamoto et al., 2003)

### **1.1.6 Toll like receptor signalling pathways**

Detection of microbial ligands by epithelial TLRs is regarded as the initial process in regulating innate immune reactions. When microbial ligands are detected, TLRs are dimerised and initiate a signalling cascade, resulting in the pro-inflammatory cytokines becoming activated. There are five adaptor proteins that arbitrate TLR signalling; MyD88, TIR-associated protein (TIRAP) or Mal, TIR domain containing adaptor protein inducing IFN- $\beta$  (TRIF), TRIF-associated adaptor molecules (TRAM), and Sterile-alpha and Armadillo motif comprising protein (SARM) (O'Neill and Bowie, 2007, Yamamoto et al., 2002). Two signalling pathways are induced by microbial ligands; MyD88-dependent activates (TLR 2, 4, 5, and 9) and MyD88-independent (TLR3 and 4) (Akira and Takeda, 2004, Takeda and Akira, 2005). MyD88 serves a crucial role as a necessary

adaptor molecule for TLR and IL-1, which connected to the ligand induced expression of IL-6. MyD88 additionally has a role in phagocytosis and the bacterial pathogens being eradicated. If this process does not occur, then the phagocytotic immune response is not able to undertake its role. MyD88 have an integral role in the initiation of specific cytokines that are responsible for the activation of TNF $\alpha$  and IL-1 that occur in receptor ligation (Inoue et al., 2007). The joining of TLR and MyD88 recruits IL-1 receptor-associated kinase 4 (IRAK-4), enabling the connection of IRAK-1. Following phosphorylation by IRAK-4, IRAK-1 joins with TNF receptor activated elements 6 (TRAF-6), a process opposed by the negative regulator, IRAK-M (Pålsson - McDermott and O'Neill, 2004). TRAF-6 activation is stimulated, transforming the PGF- $\beta$  activated kinase (TAK-1), which phosphorylates MAP kinases and the inhibitory  $\kappa$ B kinase (IKK) complex resulting in triggered IKK complexes. The initiation of IKK complexes will phosphorylate I $\kappa$ B, providing freedom to NF- $\kappa$ B from its restrictions and permitting nuclear translocation of NF- $\kappa$ B, culminating in genes for inflammatory cytokines going through transcription and co-stimulatory molecules (Banerjee and Gerondakis, 2007) (Figure 1.1-4). The array of mechanisms appears to be having an influence in regulating TLR initiation by the intestinal epithelium, as the persisting and extensive initiation of TLRs can prime uncontrolled inflammation. These processes regulate the shared results of a range of negative regulators, such as IRAK-M, Tollip, SIGIRR, A20, and NOD-2 (Shibolet and Podolsky, 2007). Tollip has been referred to as a negative regulator of NF- $\kappa$ B signalling, activated by a range of stimuli including LPS (Burns et al., 2000). The prime role of Tollip, located in the Golgi apparatus (Li et al., 2004), is to negatively regulate TLR signalling by restricting the processes of IL-1 receptor-associated kinase (IRAK). The limitations of the activity of IRAK result in restrictions to the inflammation that is connected with the up-regulation of TLR2 and TLR4 expression (Zhang and

Ghosh, 2002). Furthermore, research into animal and human cell line models additionally indicated Tollip regulates the magnitude of inflammatory cytokine production in relation to IL-1 $\beta$  (Didierlaurent et al., 2006). As such, maintaining steady intestinal homeostasis at the gut epithelial cell location necessitates a range of functions of the epithelial cell hypo-responsiveness to LPS. One of these roles does concern the expression of Tollip. Interleukin-1 receptor connected kinase (IRAK)-M is a protein that has a role in several immune and epithelial cells. IRAK-M is an efficient negative regulator of TLR signaling within MyD88, reducing the signalling via NF- $\kappa$ B and the expression of pro-inflammatory such as TNF $\alpha$  and IL-1 $\beta$  (Kobayashi et al., 2002). SIGIRR is a negative regulator that has a role within the signal transduction of several receptors of the TLRs. Overexpression of SIGIRR can limit NF- $\kappa$ B-dependent luciferase expression as controlled by TLR4 and TLR9 (Figure 1.1-4) (Zhao et al., 2012). The inhibition of cytokine signalling (SOCS) is induced by TLR, which plays an important function within the negative regulation of TLR signals (Yoshimura et al., 2007). In general, these negative regulatory molecules have the role of down-regulating the mechanisms and extent of immune reactions that present a danger to the effective functioning of the relevant immune system in question.

### **1.1.7 NOD-like receptor (NLRs)**

Cytoplasmic NLR detect an extensive array of microbial ligands and toxins in addition to particular damage-associated molecular patterns (DAMP) of the host cell. NODs and NALPs are significant groups that are suitably attributed within the NLR family (Williams et al., 2010). Nucleotide Oligomerisation domains (NODs) are a family of cytosolic proteins that function as PRRs and initiate a signalling cascade that results in an inflammatory reaction. The NLR family members NOD1 and NOD2 are activated by



varying elements of bacterial peptidoglycan. NOD1 signals via TRAF2 and TRAF5 and NOD2 signals via RIPK2 leading to NF- $\kappa$ B initiation. Additional NLRs are involved in the inflammasome being devised that results in caspase-activation and IL-1 secretion (Clarke and Weiser, 2011, Martinon et al., 2009). NOD2 was the first risk gene to be recognised to confer increased risk to CD (Hugot et al., 2001, Ogura et al., 2001). NOD-2, sometimes referred to as Caspase activating and recruiting domain (CARD15), is a component within the NOD family and identifies intra-cytoplasmic PAMPs of muramyl dipeptide (MDP), the smallest bioactive elements of PGN (Girardin et al., 2003a). NOD-2 is expressed by monocytes/macrophages, dendritic and intestinal epithelial cells when responding to intracellular microorganisms. The activation of NOD-2 by invading intracellular pathogenic microorganisms like *Salmonella* and *Shigella* comes before the amplification of inflammation process which occurs via up-regulating the generation of pro-inflammatory cytokines (Girardin et al., 2003b). That said, commensal bacteria like *Lactobacillus casei* strain *Shirota* (LcS) is additionally detected via NOD-2. However, they result in advantageous outcomes via limiting inflammatory cytokines, thus resulting in an enhanced gut mucosa within the IBD model (Matsumoto et al., 2009). NOD-2 has a pivotal function in controlling the gut mucosa (van Heel et al., 2005); NOD-2 gene polymorphism initiates defects in PGN signalling. This occurs when the recognition of commensal PGN results in amplifying the immune reaction connected to the up-regulation of the inflammatory cytokines.

Three uncommon SNPs in NOD2 are connected to vulnerability to ileal CD with an odds ratio equal to 2.4 in heterozygote people and 17.1 in homozygotes or compound heterozygotes. This can be recognised as the most significant link with IBD identified thus far (Economou et al., 2004). This connection has been widely extrapolated across

European ancestry, but no such link has been identified in Asian and African–American societies (Yamazaki et al., 2002, Kugathasan et al., 2005). Ten years on, the functional purpose of NOD2 mutations remains widely debated, yet research that has hitherto been undertaken indicates that they are loss-of-function mutations that result in diminished NF- $\kappa$ B initiation (Abraham and Cho, 2006). This insufficient reaction might lead to diminished reduced antibacterial agent generation and pathogenic microbial invasion (Wehkamp et al., 2004). Additional research indicates that the loss of the role of NOD2 might cause a reduction in the limitations of TLR2 stimulation, resulting in the initiation of inflammatory pathways and excessive Th-1 reactions (Watanabe et al., 2004c). Additionally, the distinct NOD2 3020insC has been demonstrated to limit IL-10 expression within human monocytes. This has initiated the theory that NOD2 mutations might lead to insufficient immune regulation (Noguchi et al., 2009). NOD2 single nucleotide polymorphisms have additionally been recognised in further chronic inflammatory disorders, including leprosy, Blau syndrome, an autosomal dominant chronic granulomatous inflammatory disorder that influences upon the joints, and early onset sarcoidosis (EOS). From this, it appears that NOD2 is a significant immune receptor within the process of inflammation (Zhang et al., 2009, Henckaerts and Vermeire, 2007).

Furthermore, CD has been linked with mutations in ATG16L1 and IRGM genes. These have a part in autophagy, a importantly conservative cellular practice where a double-membrane vesicle is created to surround the invading intracellular bacteria or cellular cytoplasmic material to permit effective degradation (Hampe et al., 2007, Parkes et al., 2007). Autophagy conventionally materialises because of stress or starvation to reuse crucial cellular proteins and amino acids. Whereas immune stimulation via TLRs have

been identified as initiating autophagy (Delgado et al., 2008), research conducted recently has connected NOD2 with inducing autophagy and have demonstrated that NOD2 and ATG16L1 alternatives both induce modified autophagy, antigen presentation and intracellular bacterial handling by DCs (Cooney et al., 2010, Travassos et al., 2010).

### **1.1.8 Cytokines association with IBD**

The change association of cytokine expression in inflammatory disease versus the homeostatic state is the key marker. Cytokines are intracellular messengers. They provide the signals that activate and control inflammatory reactions to pathogens and injury. The release pathway for cytokines within macrophages occur via extensive TNF signalling (Li et al., 2012). Activated macrophages generate a cascade of pro-inflammatory and/or anti-inflammatory cytokines such as TNF $\alpha$ , IL-1 $\beta$ , IL-18, IL-6, IL-10 and IL-12. TNF $\alpha$  is a multi-functional cytokine that is able to control numerous cellular and biological practices, including immune purpose, cell distinctiveness, proliferation, apoptosis and energy metabolism (Cawthorn and Sethi, 2008).

#### **1.1.8.1 Pro-inflammatory cytokine, TNF $\alpha$**

TNF $\alpha$  is a pro-inflammatory cytokine that is generated by initiation macrophages as a form of reaction to microbes, particularly in response to stimulation by LPS of Gram negative bacteria. TNF $\alpha$  is a significant mediator of acute inflammation and regulates the recruitment of macrophages to locations of infection via activating endothelial cells to secrete adhesion chemokines (Kigerl et al., 2009). TNF $\alpha$  generate chemokines, which are chemotactic cytokines, and activates the hypothalamus to result in fever. TNF $\alpha$  additionally advocates the secretion of acute phase proteins. Furthermore, it has deep autocrine influences, its predominant cellular origin, triggering them and

improving their cytotoxic prospects (Beutler and Cerami, 1988). These contrasting influences of TNF $\alpha$  might partly be due to the presence of two distinct signaling pathways mediated through TNF receptor 1 (TNFR1; p55) and TNFR2 (p75). It is reckoned that TNFR1 mediates the deaths of cells, whilst TNFR2 progresses cell death or advocates cell initiation, expansion or proliferation (Arnett et al., 2001).

Macrophages are regarded as the main aspect generating a potent pleiotropic pro-inflammatory cytokine, TNF $\alpha$ . Rather paradoxically, they are very sensitive to this process themselves (Vassalli, 1992, Parameswaran and Patial, 2010). In fact, it has been indicated that TNF $\alpha$  has an important function in controlling the inflammatory reactions of macrophages (Parameswaran and Patial, 2010). Overall, the initiation of macrophages and TNF $\alpha$  generation materialise through an array of activatory signals. The LPS-mediated signalling pathway is one of these instances (Sweet and Hume, 1996, van der Bruggen et al., 1999). LPS signalling happens via LPS-binding protein (LBP) in plasma (Ulevitch and Tobias, 1995). Hence, the LBP/LPS complex joins the surface bound receptor of CD14 on monocyte/macrophage (Tobias et al., 1993, Delude et al., 1994). LPS is subsequently transmitted to a signalling transmembrane molecule, TLR4 (Beutler and Rietschel, 2003). The TLR4/LPS joining results in the initiation of differing intracellular pathways, including the NF- $\kappa$ B pathway that is initiated by MyD88 and IRAK. This controls the phosphorylation and ubiquitination of I $\kappa$ B $\alpha$ . Resultantly, I $\kappa$ B $\alpha$  is dispelled and NF- $\kappa$ B is activated (Chen et al., 2005, Kawai and Akira, 2007, Karin, 2009). Active NF- $\kappa$ B (p50/p65) is ultimately translocated into the nucleus (Sun and Ley, 2008). As such, LPS-induced NF- $\kappa$ B activation leads to the secretion of the TNF $\alpha$  gene expression in macrophages (Shakhov et al., 1990). Following such signalling cases, a pro forma form of II transmembrane TNF $\alpha$  containing a molecular mass of 26 KDa is

generated. This manifests itself as a membrane bound precursor that can be proteolytically cleaved by TACE (TNF- $\alpha$ -converting enzyme), a disintegrin and metalloproteinase (ADAMs) (Black et al., 1997, Moss et al., 1997, Peschon et al., 1998). Furthermore, the way in which TNF $\alpha$  expression is induced is additionally activated by networks of signalling pathways like mitogen-activated protein kinase (MAPK) pathways incorporating extracellular signal-regulated kinases (ERK 1, 2), p38 and c-Jun N-terminal kinase (JNKs). Further transcription aspects include initiated protein-1 (AP-1) (Tsushima and Mori, 2001, Beutler and Rietschel, 2003, Sun and Ley, 2008). The initiation of JNKs (Swanek et al., 1997), p38 and ERK1, 2 (Geppert et al., 1994, Lee and Young, 1996) in RAW 264.7 is essential for LPS-induced TNF $\alpha$  generation. Furthermore, NF- $\kappa$ B can be triggered independently of the MyD88 initiation pathway via protein kinases C (PKC  $\alpha$  and  $\beta$ ) when reacting to LPS and PMA stimulation (Martin et al., 2003, Foey and Brennan, 2004). This said, TNF $\alpha$  is necessary for the role of macrophage immune and host defence, with unrestricted TNF $\alpha$  generation occasionally causing tissue damage (Parameswaran and Patial, 2010). For example, extensive generation for TNF $\alpha$  was revealed to detrimentally impact upon the pathology of inflammatory disorders as in autoimmune conditions as extended TNF $\alpha$  secretion results in tissue disruption being caused within the rheumatoid arthritis (Drexler et al., 2008) or an extreme reaction to infectious agents in sepsis (Conte et al., 2006, Smith et al., 2006). As such, a controlling function must develop to diminish the extreme generation of pro-inflammatory TNF $\alpha$ . This might be beneficial in forming therapeutic possibilities for disease-associated inflammation.

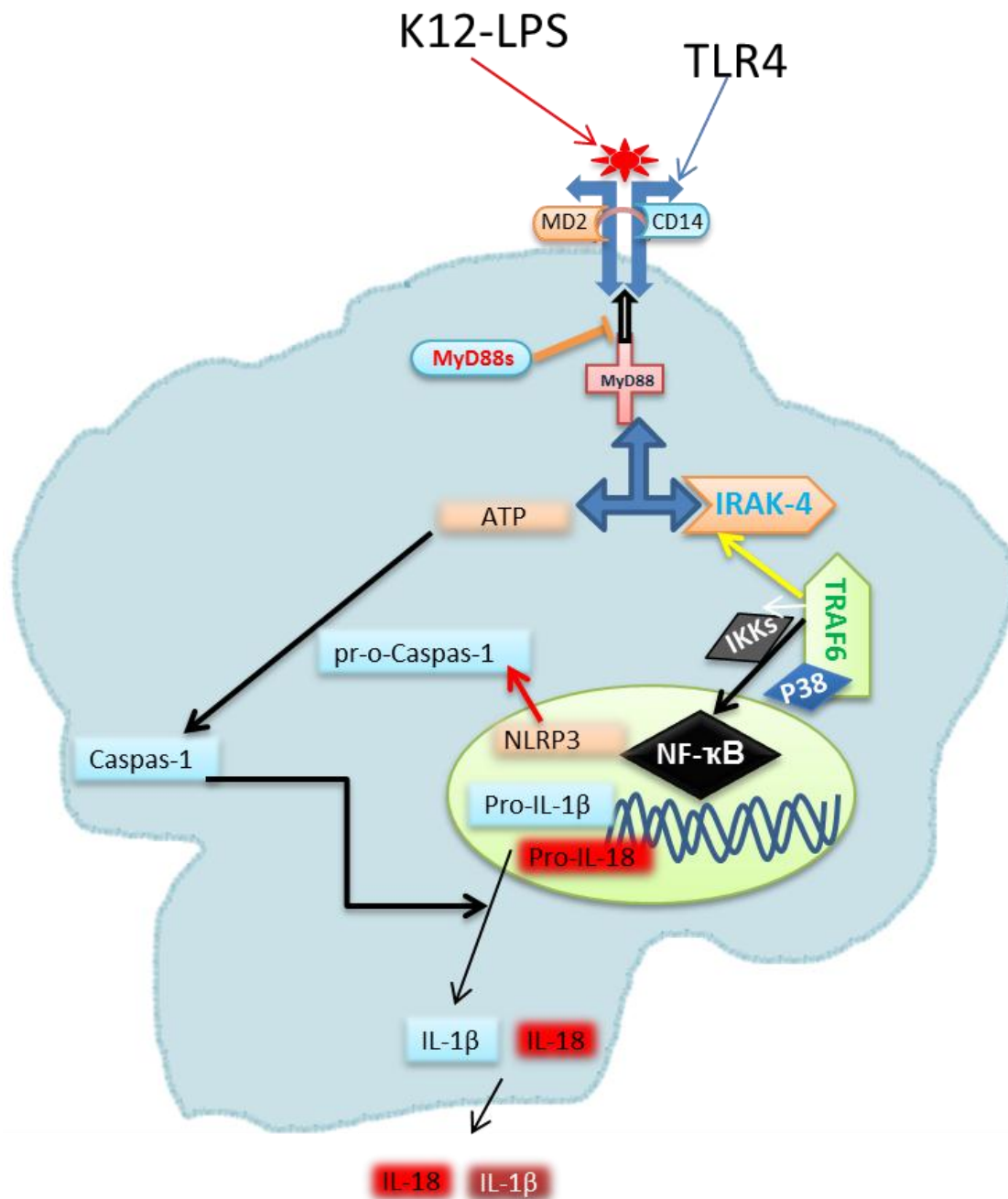
### **1.1.8.2            Inflammasome activation and IL-1 $\beta$ & IL-18**

The initiation of neutrophils and macrophages to overwhelm pathogens and to free toxic oxygen and nitrogen radicals relies on pro-inflammatory cytokines like IL-1 $\beta$  and IL-18 that are mainly generated by monocytes/ macrophages (Dinarello, 1996, Dinarello, 1999). The inactive precursors of IL-1 $\beta$  and IL-18 (pro-IL-1 $\beta$  and pro-IL-18) differ from other pro-inflammatory cytokines in that they depend upon cleavage into the bioactive cytokines via an enzymatic process. One of the most significant enzymes in determining the activation of pro-IL-1 $\beta$  and pro-IL-18 is the intracellular cysteine protease caspase-1 (Dinarello, 1996). Multimeric protein platforms called the inflammasome are important regulators of this process (Latz, 2010). The inflammasome characteristically comprises a nucleotide joining domain leucine-rich repeat (NLR) protein, the adaptor molecule apoptosis-associated speck-like protein, which comprises a CARD (ASC) domain, and caspase-1. Four inflammasome complexes have been referenced hitherto include NLRP3, NLPC4 etc. The inflammasome resulting from the NLR family member Nlrp3 and the adapter protein ASC is the most widely studied. It is possible to initiate the Nlrp3 inflammasome via an important quantity of stimuli. Some have a bacterial foundation (MDP, bacterial RNA and double-stranded RNA) whilst some are danger -associated molecular trends (DAMPs including uric acid crystals and amyloid- $\beta$ ). Furthermore, exogenous compounds including asbestos, silica or alum adjuvant have been referenced as initiating the Nlrp3 inflammasome (Martinon et al., 2004, Dostert et al., 2008, Kanneganti et al., 2006, Eisenbarth et al., 2008). Whilst advances have been achieved in raising our awareness of the control of IL-1 $\beta$  processing, contention is still evident in terms of the ability of TLR ligands, for instance LPS, to initiate caspase-1 and free IL-1 $\beta$ . The monocyte-like leukemia cell line THP-1 does generate dynamic IL-1 $\beta$  when reacting to LPS stimulation. Some have

argued that the mere presence of LPS is not sufficient to activate IL-1 $\beta$  release (Martinon et al., 2004). However, the findings from research indicate that IL-1 $\beta$  exudes from monocytes after a single stimulation with a TLR ligand (Dinarello et al., 1986, Dinarello et al., 1987). Research has recently provided new insights in this debate, suggesting that the synthesis and release of IL-1 $\beta$  vary across human monocytes and macrophages (Netea et al., 2009). Monocytes have the capacity to initiate caspase-1 by autocrine generation of ATP. Thus, stimulation with a TLR ligand can swiftly result in active IL-1 $\beta$  in monocytes being released via the induction of IL-1 $\beta$  transcription. This does not rely on the requirement for a second signal from the environment to initiate caspase-1 (Figure 1.1-3). However, macrophages, dendritic cells and the monocytic cell line THP-1 depend upon two particular signals to instigate IL-1 $\beta$  transcription and translation and to activate caspase-1. These two signals will consequently produce IL-1 $\beta$  processing and secretion (Figure 1.1-3) (Netea et al., 2009). Whilst caspase-1 is evident in freshly acquired human blood monocytes, secretion of dynamic IL-1 $\beta$  remains reliant on inflammasome aspects, especially ASC (Netea et al., 2009). In research conducted of turpentine-induced inflammation, IL-1 $\beta$   $-/-$  mice face defence against inflammation but caspase-1-deficient mice are not (Fantuzzi et al., 1997, Horai et al., 1998). Additionally, caspase-1 seems to be dismissed when defending against particular forms of microorganisms, like *Chlamydia trachomatis*. That said, IL-1 $\beta$  affects the inflammatory reaction induced by these microorganisms (Cheng et al., 2008, Prantner et al., 2009). The findings indicate that caspase-1 and inflammasome initiation is vital within a few but not every form of IL-1 $\beta$ -driven inflammation. Caspase-1 initiation is only one role for cleavage of IL-1 $\beta$  and IL-18, and hence the presence of contrasting pathways of IL-1 $\beta$  and IL-18 initiation, which do not rely on the inflammasome, must be recognised. Additionally, there is not much chance that a

whole form of trend identifying receptors have developed to merely be used for initiating caspase-1. Consequently, the objective should be set of further investigating the roles of NLRs that surpass the function they play in instigating caspase-1. In the pathophysiology of IBD, interleukin-1 is a dominant component in the regulation of inflammation in the mucosal gastrointestinal tract (Dukovich et al. 1986). It has been reported that IL-1 $\beta$  levels increase in gut tissue in intestinal inflammation of animal models and in the mucosa of IBD patients (McCall et al. 1994; Reinecker et al. 1993). IL-18 has been reported to be upregulated in IBD patients as it is capable to stimulate IL-1 $\beta$  as well as IL-8 from activated macrophages, consequently modulating the concluding pathway of CD immune pathogenesis. Therefore, it is possible that IL-18 will be the essential as a primary initiating cytokine in Th1-mediated diseases, such as CD (Pizarro et al., 1999).





**Figure 1.1-3: Signalling events associated with IL-1 $\beta$  and IL-18 production, inflammasome activation.**

TLR4/MD2 signal through either MyD88 to activate NF- $\kappa$ B. Activation of NF- $\kappa$ B results in increased transcription of *nlrp3*. Inflammasome activation occurs in response to such agonists as extracellular ATP. If TLR4 signaling increases NLRP3 protein levels such as with MyD88-competent LPS then the activation event permits the newly expressed NLRP3 pro-caspase-1, leading to caspase-1 processing to its active form and inflammasome assembly. Pro-IL-1 $\beta$  and pro-IL-18, which is associated with the inflammasome, is processed to mature IL-1 $\beta$  and IL-18 and secreted into the extracellularly. Adapted from (Chilton et al., 2012)

IL-1 is an inflammatory cytokine that functions in a way that has similarities with TNF $\alpha$ . The IL-1 process comprises two agonists, IL-1 $\alpha$  and IL-1 $\beta$ , a particular initiation process (IL-1-converting enzyme, or ICE, caspase 1), a receptor antagonist (IL-1ra) which has varying isoforms, and two high affinity surface-binding molecules (Sims et al., 1993). IL-1 $\beta$  is secreted into localised interstitial fluid and blood circulation within inflammation and governs extensive pro-inflammatory processes. IL-1 $\beta$  affects the activation of the inflammatory reaction. Evidence indicates that IL-1 $\beta$  has a significant function within the pathogenesis of intestinal inflammation in IBD. In animal models of intestinal inflammation, IBD individuals have enhanced forms of IL-1 $\beta$  in their intestinal tissue (Ligumsky et al., 1990, Spiller et al., 2000, Casini-Raggi et al., 1995, Isaacs et al., 1992). There is a link between developing forms of IL-1 $\beta$  and the extent of intestinal inflammation which has been proven (Reinecker et al., 1991). An imbalance between IL-1 $\beta$  and its antagonist IL-1ra is present within the intestinal mucosa of IBD patients, indicating that the limited anti-inflammatory types of IL-1 to oppose the enhanced extent of IL-1 $\beta$  might be a significant pathogenic shortcoming (Cominelli et al., 1990). In line with this prospect, the way in which the rIL-1ra was administered helped to avoid the intestinal inflammation in a rabbit model of colitis (Cominelli et al., 1992). Research has also indicated the presence of IL-1 $\beta$  gene polymorphisms in IBD individuals that impacts the course and the extent of increase intestinal inflammation (Nemetz et al., 1999).

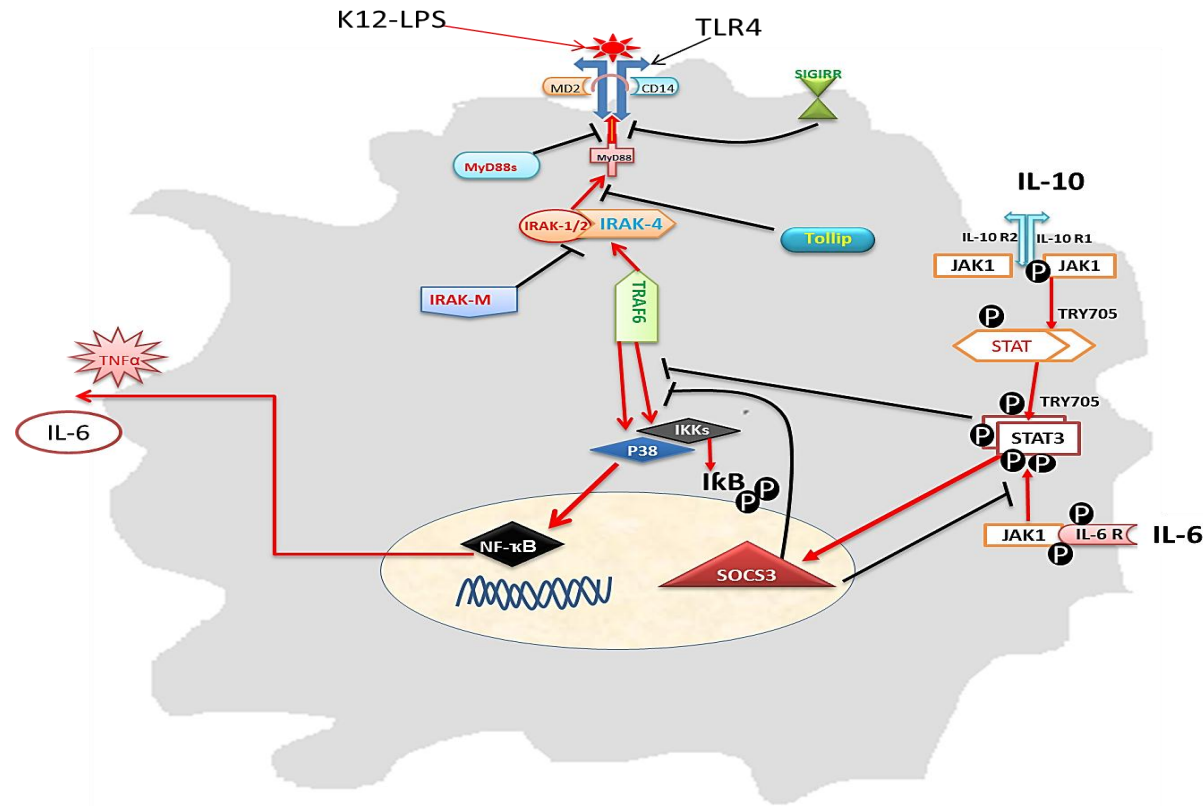
Interleukin-18 was initially outlined in 1989 as “IFN $\gamma$ -inducing factor” which was remote within the serum of mice after an intraperitoneal injection of endotoxin. Subsequently, it was referred to as IL-18. Unexpectedly, the emerging cytokine was connected to IL-1 and especially to IL-1 $\beta$ . In a similar manner to IL-1 $\beta$ , IL-18 is still an

intracellular cytokine (Dinarello et al., 2013b). Various human autoimmune conditions are linked to enhanced generation of IFN $\gamma$  and IL-18. These disorders include systemic lupus erythematosus, rheumatoid arthritis, Type-1 diabetes, Crohn's disease, psoriasis, and graft versus host disease, all of which are perceived to be at least partly governed by IL-18 (Dinarello et al., 2013b). After cleavage by caspase-1, mature IL-18 is secreted from the monocyte/macrophage. However, in excess of 80% of the IL-18 precursor is not processed within the cell (Fantuzzi et al., 1999). Interleukin-18 displays attributes of alternative pro-inflammatory cytokines, including advances in cell adhesion molecules, nitric oxide synthesis, and chemokine generation. Opposing IL-18 activity diminish metastasis within a mouse model of melanoma; this is because of a decline in IL-18-induced expression of vascular cell adhesion molecule-1 (Vidal-Vanaclocha et al., 2000). Because IL-18 can increase IFN $\gamma$  generation, opposing IL-18 activity in autoimmune disorders is an appealing therapeutic objective because anti-IL-12/23 limits this variety of Crohn's disease in addition to psoriasis. There is a probability that IL-18, being constitutive in the intestinal epithelium (Pizarro et al., 1999), has a regulatory function because the cytokine results in the maintaining the intestinal barrier. With the loss of the barrier, the microbial products activate macrophages in the lamina propria and caspase-1 dependent generation of IL-18 outcomes in inflammation. In this particular instance, restriction of IL-18 generation in caspase-1-deficient mice or wild-type mice being treated with neutralizing anti-IL-18 antibodies or caspase-1 inhibitors is protective (Dinarello et al., 2013b).

#### **1.1.8.3 IL-6 and IL-10**

IL-6 is a pleiotropic cytokine that has a significant influence in inflammation, immune control, hematopoiesis, and oncogenesis (Hirano et al., 1990, Kishimoto, 1989).

Research has increasingly suggested that an IL-6 signalling pathway shows a crucial part in the uncontrolled intestinal inflammatory activity of IBD (Atreya and Neurath, 2005, Ishihara and Hirano, 2002) . IL-6 works as both a pro and anti-inflammatory cytokine (Kigerl et al., 2009). It is secreted by T cells and macrophages to initiate a response from the immune system to injury, particularly burns or other tissue damage. IL-6 has a crucial function in forming defence towards bacteria, such as *Streptococcus pneumonia* (Chapman et al., 2013). IL-6's role, as an anti-inflammatory cytokine, is activated via its inhibitory influence on TNF $\alpha$  and IL-1 (Chapman et al., 2013). In the intestinal mucosa, IL-6 has been identified in macrophages, lymphocytes such as B cells and T cells, and intestinal epithelial cells (Stevens et al., 1992, Jones et al., 1993, Kusugami et al., 1995). Research has indicated signal transducer and activator of transcription 3 (STAT3) as a downstream signaling pathway of IL-6 (Alonzi et al., 2004). Advanced rates of phosphorylated STAT-3 was connected with the disorder activity in individuals suffering from IBD as well as in some animal models of colitis or enteritis (Suzuki et al., 2001). STAT-3 activation, especially in mucosal T cells, is a significant inflammatory event within the progression of IBD. SOCS genes are involved in negative regulation of the JAK/STAT pathway induced by cytokine signaling (Yoshimura et al., 2003). Present thought is that SOCS3 and STAT-3 are both extensively expressed within human IBD and in animal IBD models (Suzuki et al., 2001). The levels of SOCS3 in the inflamed intestine are probably inadequate to prevent STAT-3 activation, thus inducing chronicity of inflammation (Mitsuyama et al., 2006) (Figure 1.1-4).



**Figure 1.1-4: Association between TLR4 signalling pathway and JAK/STAT signaling pathway.**

*E.coli* Lipopolysaccharide LPS (K12-LPS); TLR4, Toll-like receptor 4; TLR2, Toll-like receptor 2; CD14; MD2, myeloid differentiation protein-2; MyD88, myeloid differentiation primary response 88; MyD88s, myeloid differentiation primary response 88 short version; IRAK-1,2,4 &M, IL-1 receptor-associated kinase; Tollip, Toll interacting protein; TRAF6, TNF receptor-associated factor-6; SIGIRR, Single Immunoglobulin IL-1 Receptor Related molecule; IKKs, IκB kinases; NF-κB, nuclear factor kappa B; P38, P38 mitogen-activated protein kinases; SOCS3, Suppressor of cytokine signaling proteins. As adapted from (Zhu et al., 2015, Cavaillon and Adib-Conquy, 2006a, Wang et al., 2011).

In contrast, activated macrophages and Th2 cells generate IL-10, and it is primarily an inhibitory cytokine or anti-inflammatory cytokine. It restricts the generation of IFN- $\gamma$  by Th1 cells, which transition the reactions of the immune system towards a Th2 type. IL-10 restricts the generation of cytokines by activated macrophages and the expression of MHC II on macrophages, which leads to immune reactions being diminished (Walsh et al., 2013). Transformation growth factor beta (TGF $\beta$ ) arises from proteins generated by an array of cell forms that regulate the proliferation of cellular variation. It is generated by numerous cell forms and macrophages where it materialises on their surface bound to CD36 (Yoshimura et al., 2007). Extensive levels of TGF- $\beta$  are evident within the majority of tissues (Bensinger and Tontono, 2008). IL-10 restricts the generation of TNF $\alpha$  and macrophage inflammatory protein-2 (MIP-2) and maintains hemodynamic boundaries (Ding et al., 2003). *In vitro* research has indicated that IL-10 restricts the release and role of IL-1 $\beta$ , IL-6, TNF $\alpha$ , granulocyte-macrophage colony-stimulating factor, and IL-12 (Cassatella et al., 1993, de Waal Malefyt et al., 1991), consequently implying a conventional endogenous feedback tool for the regulation of immune outcomes and inflammation. Furthermore, *in vivo* research has indicated the direct suppressive impact of IL-10 on pro-inflammatory outcomes (Asadullah et al., 1998). Activation of STAT-3 is crucial for the entirety of the acknowledged outcomes of IL-10 (Lang et al., 2002, Takeda et al., 1999). IL-10 joins to its cognate receptor (IL-10R), a tetramer comprised of two notably specific chains (IL-10R1 and IL-10R2). As such, the findings of IL-10 appear to be restricted to particular cells of the immune system. IL-10 joining to IL-10R initiates the IL-10/JAK1/STAT-3 cascade, where phosphorylated STAT-3 homodimers translocate to the nucleus in a matter of seconds to initiate the target genes being expressed (Hutchins et al., 2013).

#### 1.1.8.4 Endotoxin Tolerisation (ET)

Immune destruction can result from immune activation or deactivation mechanisms. ET involves cells undergoing a process involving a transient unresponsive condition, and not being able to react to additional obstacles with endotoxin (e.g. LPS), after encountering a particular extent of endotoxin. ET has been researched *in vivo* and *in vitro* in both humans and animals (Biswas and Lopez-Collazo, 2009). Suppression of pro-inflammatory reactions result in autoimmunity and cancer within the host (Foster and Medzhitov, 2009). In contrast, anti-inflammatory restriction is against the pathogen. Gut mucosal MΦs are necessary for ET due to their distinctiveness and initiation process. ET induced by MΦ is present in varying ways, such as the launch of anti-inflammatory cytokines (e.g. IL-10 and TGFβ), the down-regulation of PRRs (e.g. TLR4) or the shedding of cytokine receptors and PRRs and /or induction of negative regulatory molecules, like Tollip, Myd88s, SARM, sTLRs, sCD14 and SIGIRR (O'Neill, 2008) also reviewed in (Foey and Crean, 2013).

The immune-suppression of anti-inflammatory M2 MΦ characterise up regulation for anti-inflammatory cytokine (such as IL-10) and present down regulation for pro-inflammatory cytokines (such as TNFα) (Mantovani et al., 2005). Yet, it is excessively simplistic to allocate endotoxin tolerisation phenomenon to a specific MΦ subset due to the varieties of MΦ phenotypes in their variation, activation and tolerisation state (Cavaillon and Adib-Conquy, 2006b). The initial *in vitro* research of ET in human monocytes (Cavaillon and Adib-Conquy, 2006b, del Fresno et al., 2009) has revealed that the most restricted genes in terms of re-stimulation with LPS were the pro-inflammatory cytokines and chemokines like TNFα, IL-6, IL-1β, CCL3 and CCL4 (del Fresno et al., 2009, Draisma et al., 2009, Foster and Medzhitov, 2009, Mages et al.,

2007). However, genes of anti-inflammatory cytokines such as IL-10 and TGF $\beta$  and negative regulators like IRAK-M were up regulated (Biswas and Lopez-Collazo, 2009).

Research has indicated that initiation of tolerance is associated with suppression of TLR expression (Nomura et al., 2000, Wang et al., 2002). Down-regulation of downstream immune reactions is connected with diminishing expression of TLR4 surface protein and the lack of the necessary co-receptor subunits MD2 and CD14 (Abreu et al., 2001, Cario and Podolsky, 2000b, Otte et al., 2004). Additionally, MD-2B, an alternatively spliced type of MD-2 might restrict the dynamic type of MD-2 via competing for joining to TLR4, culminating in LPS tolerance (Otte et al., 2004). It has been recognised *in vitro* research that there are shortcomings in the TLR4 pathway in ET. That is, there appears to be a link between ET and the decline of TLR4-MyD88 complex progression, elimination of IRAK-1 activity, and disturbance in NF- $\kappa$ B initiation (Biswas and Lopez-Collazo, 2009, Fan and Cook, 2004). While the heterodimers p65/p50 NF- $\kappa$ B is activating the TNF $\alpha$  and IL-12 expression in the early stages of inflammation, the homodimers p50/p50 NF- $\kappa$ B in ET would tolerise the expression of these genes and enhance the expression of the IL-10 and TGF $\beta$  genes instead. Yes, this basis stemmed from the argument that the plasticity of NF- $\kappa$ B role within varying stages of inflammation might acquire separate phenotypes from inflammatory to anti-inflammatory reviewed in (Biswas and Lopez-Collazo, 2009).

As well as TLRs, the cytoplasmic nucleotide-binding oligomerisation domain (NOD) proteins, NOD1 and NOD2, might have a role within the natural immune reaction (Cario and Podolsky, 2005). It has been proposed that NOD2 might govern the TLR-signalling pathway in a detrimental manner. As such, restriction of TLR2-driven Th1-cytokine reactions by targeting NF- $\kappa$ B initiation seemed to arise from stimulation with



the NOD2 ligand, MDP. Netea et al, 2004, discovered that stimulation with peptidoglycan stimulation or synthetic lipopeptide stimulation, given the lack of NOD2 CARD15 mutation in Crohn's Disease, might result in NF- $\kappa$ B dysregulation and the subsequent inconsistency of cytokine generation (rise of IL-12, reduction of IL-10) through TLR2 (Netea et al., 2004, Watanabe et al., 2004b) possibly having an influence on the pathogenesis of Crohn's Disease (Cario and Podolsky, 2005). The removal of MΦs and the restriction of pro-inflammatory cytokines in ET are frequently connected with the expansion in anti-inflammatory cytokine generation, like IL-10 and TGF $\beta$  (Cavaillon and Adib-Conquy, 2006a, Schroder et al., 2003, Sfeir et al., 2001). Endogenous suppressors like IRAK-M, ST2, and short version of MyD88 (MyD88s) are connected with TLR4 downregulation and an absence of LPS reaction. IRAK-M expression has been suggested and verified within both human and mice ET models (Biswas and Lopez-Collazo, 2009).

Extensive research has utilised *in vitro* and *in vivo* concepts to develop our awareness of ET. Foey and Crean (2013) indicate that MΦ subsets, resulting from the THP-1 cell line, demonstrated varying reactions to PG-LPS activation when they were pre-treated with *Porphyromonas gingivalis* LPS (PG-LPS). In other words, pre-stimulation with PG-LPS of M1-like MΦs did not restrict TNF $\alpha$ , IL-6 or IL-1 $\beta$  generation when treated with PG-LPS. In contrast, it did restrict the generation of TNF $\alpha$  and IL-6 but not IL-1 $\beta$  by M2-like MΦ when treatment with PG-LPS was applied. PG-LPS-stimulated M1 CD14<sup>hi</sup> MΦs expressed a greater degree of TNF $\alpha$ , IL-1 $\beta$  and IL-6 in contrast to M1 CD14<sup>lo</sup> MΦs. However, PG-LPS- stimulated M2 CD14<sup>lo</sup> MΦs generated an enhanced extent of TNF $\alpha$ , IL-1 $\beta$  and IL-6 in contrast to the M2 CD14<sup>hi</sup> MΦs (Foey and Crean, 2013).

An additional piece of research by Sun et al (2014) placed its emphasis on the THP-1 cell line. They discovered that the level of secreted TNF $\alpha$  and IL-1 $\beta$  following retreatment by either PG-LPS or *E. coli* -LPS was beneath the extent of secreted cytokines following stimulation only once. In contrast, IL-10 level was enhanced. Sun additionally discovered that utilising the identical stimulus (homo-tolerance) resulted in the degree of TNF $\alpha$  and IL-1 $\beta$  being reduced more than (hetero-tolerance) (Sun et al., 2014).

In contrast, Foey and Crean (2013) asserted a separate theory in relation to the notion of (hetero-tolerance). Cross-tolerisation has been identified across varying microbial species, their PAMPs and the analogous PRRs, (Foey and Crean, 2013). Numerous research has presented cross-tolerisation between HK-PG and PG-LPS, HK-PG and PG-LPS +LTA, PG-LPS and *E. coli* K12-LPS and furthermore PGN tolerisation (Foey and Crean, 2013, Sun et al., 2014). Additionally, the cross tolerisation between TLR4 and NOD2, which is linked with the enhanced vulnerability of one suffering from Crohn's disease, or LPS and MDP has been asserted in (Sun et al., 2014). The argument has been proposed that NOD2 might control the TLR-signaling pathway in a detrimental manner. As such, the restriction of TLR2-driven cytokine reactions by targeting NF- $\kappa$ B instigation seemingly arises from the stimulation with the NOD2 ligand MDP. Netea et al (2004) concluded that stimulation with peptidoglycan stimulation or synthetic lipopeptide stimulation, in the absence of NOD2 such as CARD15 mutation in Crohn's Disease, could result in NF- $\kappa$ B dysregulation and a following discrepancy of cytokine secretion (growth of IL-12, decline of IL-10) through TLR2 (Netea et al., 2004, Watanabe et al., 2004b) possibly influencing the pathogenesis of Crohn's Disease (Cario and Podolsky, 2005).

One piece of research indicated that the induction of ET is to an extent dictated by the processes of NF- $\kappa$ B and the expression of CD14 (Foey and Crean, 2013). The up-regulation of the negative regulatory molecules (e.g. IRAK-M, ST2, MyD88s and SIGIRR) indicated an element of ET of NF- $\kappa$ B-dependent (Biswas and Lopez-Collazo, 2009, O'Neill, 2008).

*E. coli*-LPS has the capacity to induce ET in MΦs. While PG-LPS is generally identified by TLR2 more than TLR4, there have been arguments that *E. coli*-LPS is primarily dictated by TLR4 instead of TLR2 (Foey and Crean, 2013). Overall, it is still not known in the molecular foundation regarding how macrophages transition between an inflammatory and an immunosuppression phase (Biswas and Lopez-Collazo, 2009).

## **1.2 Aim and objective**

This research study aimed to investigate the immune modulating effect of entrapathogen *E. coli* K12-LPS on macrophage immune responses in homeostatic and inflammatory environment by developing the THP-1 cell line in generating MΦ-like subsets as a model for the current study. Initially, production of pro- and anti-inflammatory cytokines was investigated in resting and activated macrophages derived from THP-1 cells, differentiated with either PMA, to generate M1-like macrophages, or vitamin D<sub>3</sub>, to generate M2-like macrophages. To understand more about MΦ subset regulation, the intention was, firstly, to establish cytokine profiles upon stimulation of MΦ subsets by either K12-LPS as well as other PAMPs such as PG-LPS, LTA and PGN (homeostatic environment) or pro-inflammatory cytokines, TNF $\alpha$  and IL-1 $\beta$ , (inflammatory environment) and to study the effect of *E. coli* LPS on inflammasome

activation in macrophage subsets (reported in Chapter 3). Secondly, to investigate the effect of endotoxin tolerisation on MΦ subsets, PRRs and negative regulatory molecules involved in ET, IL-10 effect on K12-LPS induced pro-inflammatory cytokine production in MΦs and to study the cross-tolerance in response to different PAMPs include K12-LPS, PGN and LTA (Chapter 4). Finally, to explore the modulation induced by K12-LPS (homeostatic environment), pro-inflammatory cytokines (TNFα, IL-1β) (inflammatory environment) and ET on MΦ subsets in co-culture model considering their effect on epithelial cells relative to intestinal mucosal MΦ interacting with the epithelial cells (Chapter 5).

### 1.3 The research plan of experiments.

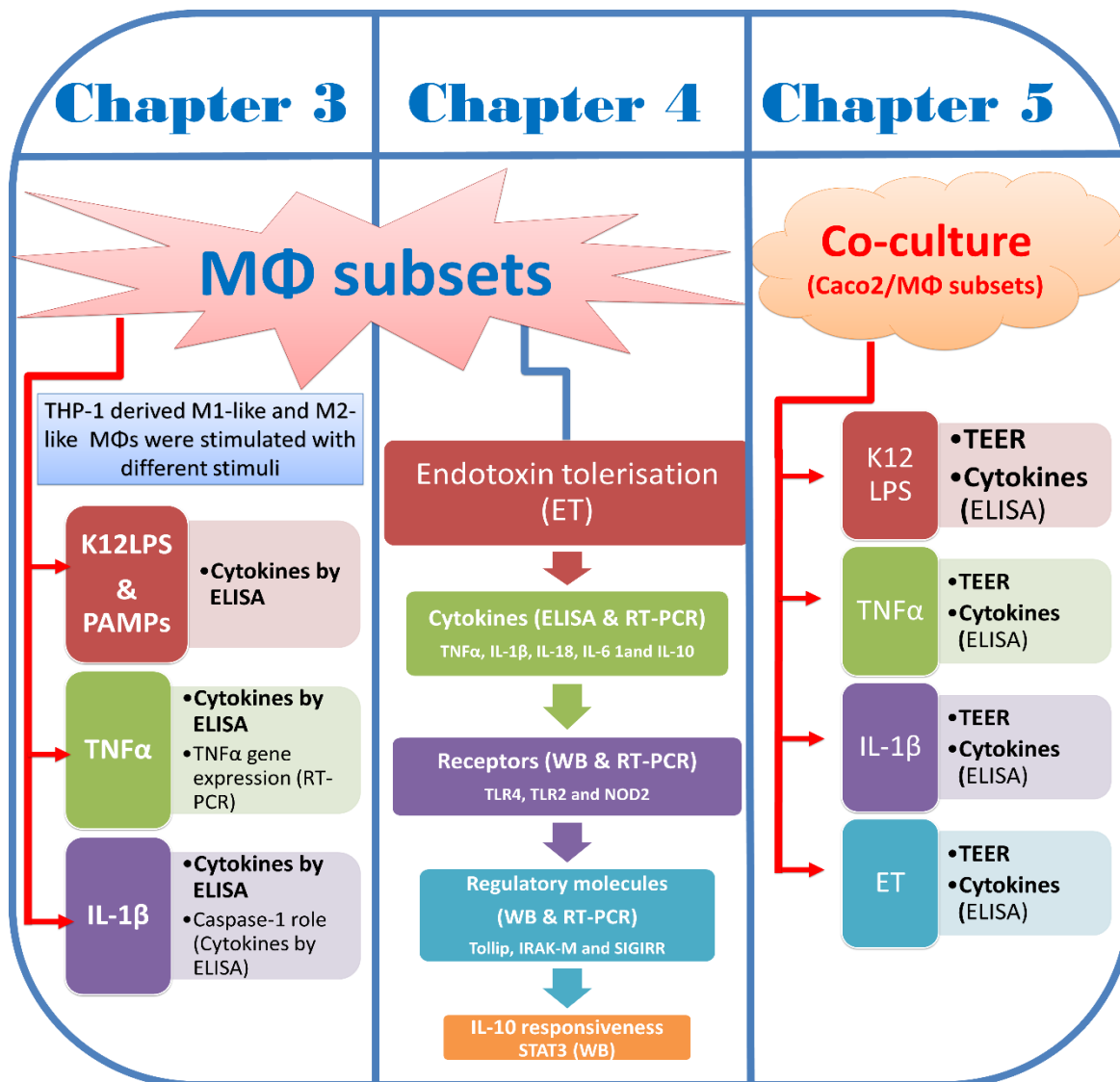


Figure 1.3-1: The research investigation plan for the whole project chapter 3, chapter 4 and chapter 5.

## **Chapter 2: Materials and Methods**

## **2.1 General Material**

Refer to Appendix 1.

## **2.2 Methodology**

### **2.2.1 Human cell culture methods**

All reagents and Antibodies used for tissue culture experiments are listed in appendix 1, table I-1.

#### **2.2.1.1 THP-1 human monocytic cell line**

Human pre-monocytic cell line, THP-1 cell line, was obtained from European collection of cell culture (ECACC, UK) and routinely used for this research. THP-1 cells were grown in Roswell park memorial institute-1640 medium RPMI-1640 medium supplemented with 10% v/v foetal calf serum (FCS), 2mM L-glutamine and 100U/ml Penicillin/ 100 µg/ml Streptomycin (R10). The cells were incubated at 37 °C and 5% CO<sub>2</sub> in incubator and sub-cultured every 3-4 days at a ratio of 1:4. Passages 7 to 32 were used for experiments (Foey and Crean, 2013).

##### **2.2.1.1.1 Caco2 intestinal epithelial cell line**

Human colon adenocarcinoma cell line (Caco2 epithelial cells) were cultured in Dulbecco's Modified Eagles' Medium DMEM medium supplemented with 10%v/v FCS, 2 mM L-glutamine and 100 U/ml penicillin, 100 µg/ml streptomycin (D10). Cells were plated out at a density of  $5 \times 10^5$  cells/ml/well in transwell, 12 well pore size 0.4 µm (Greiner bio-one, Stonehouse UK) plates and incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C for 21 days for full cell differentiation (Habil, 2013, Hilgers et al., 1990). Caco2 were sub-cultured every 3 days or once achieving 70% confluence. This process

was performed by removing the old medium followed by washing cells twice with Dulbecco's Phosphate-Buffered Saline (DPBS) and detaching cells by adding 2-3 ml of 0.25% v/v versene/trypsin EDTA (TE). Then, cells were incubated for 10-15 min at 37 °C. The cells were checked during the time of incubation by light microscopy, and gently agitated until detached. To deactivate Trypsin, D10 medium was added (Habil, 2013) and cells re-suspended in a suitable volume of D10 medium before seeding according to the designed experiment.

#### **2.2.1.2 Determination of total cell number and cell viability**

After each passage, evaluation of the number of cells and cell viability was checked. This was done by staining Ten microliters of cell suspension were mixed with 90 µl of 0.1% (v/v) Trypan blue and counting using a Neubauer haemocytometer. Furthermore, cells were stimulated with PAMP's and viability was assessed afterwards.

#### **2.2.1.3 Storage of human cell lines**

##### **2.2.1.3.1 Storage of THP-1 cell line**

THP-1 cells were centrifuged at 1200 rpm for 5 min then the supernatant was discarded and the cell pellet was suspended in 1 ml of cryopreservation media (see Table1-1 appendix1) afterwards the cells were frozen and stored at -80 °C or liquid Nitrogen.

##### **2.2.1.3.2 Storage of Caco-2 cell line**

Caco-2 cells were developed to 80% confluence trypsinised, and harvested by centrifugation at 1200 rpm for 5 min, the supernatant was removed, and cell pellets was re-suspended gently in 1 ml of storage medium and then stored at -80 °C or liquid Nitrogen.



## **2.2.2 Immune cell differentiation**

### **2.2.2.1 Macrophage cell differentiation**

M1-like Pro-inflammatory and M2-like anti-inflammatory MΦ subsets were generated by differentiation of THP-1 cells in the presence of 25ng/ml phorbol-12-myristate acetate (PMA) for 3 days for M1-like or 10nM 1,25-(OH)<sub>2</sub>Vitamin D<sub>3</sub> for 7 days for M2-like MΦ, respectively (Foey and Crean, 2013, Schwende et al., 1996). M1-like was washed out after 3 day to remove PMA and incubated for one more day prior to use in experiment.

### **2.2.2.2 Epithelial cell differentiation**

Caco2 cells differentiation into the small intestine enterocyte-like cells were achieved by long term culturing for 21 days in D10 medium (Amano and Oshima, 1999, Habil, 2013) then they were used depending on designed experiment.

## **2.2.3 Activation of macrophage cell subsets and epithelial cells**

### **2.2.3.1 Macrophage subset stimulation**

THP-1 derived M1- and M2-like MΦs were stimulated by the bacterial pathogen associated molecular patterns (PAMPs); TLR4 ligand; *E. coli* K12-LPS [0.001 – 1μg/ml], TLR2/4 ligand; PG-LPS [0.001 - 1μg/ml], TLR2 ligand; Lipoteichoic acid LTA [0.01 - 10 μg/ml] and NOD1/2 ligand; Peptidoglycan PGN [0.01- 10 μg/ml] and incubated for 18h at 37 °C/5% CO<sub>2</sub>. Afterwards, cells were harvested and supernatants stored at -20 °C until required for cytokine assay by enzyme-linked immunosorbent assay, sandwich (ELISA).

THP-1-derived M1- and M2-like MΦs were stimulated by 100 ng/ml K12-LPS at 37 °C/5%CO<sub>2</sub> for different time periods ranging from 30 min to 48 h; unstimulated cells

were used as control. The final density of cells was  $1 \times 10^6$  cells/ml plated out in 12 well, flat-bottomed tissue culture plates. Cells were harvested and supernatants and lysates were stored at  $-20^{\circ}\text{C}$ . Supernatants were required for cytokine assay by sandwich ELISA and the cell lysates were used for detection of gene expression by Real Time polymerase chain reaction (RT-PCR).

#### **2.2.3.2 Macrophage subset tolerisation**

THP-1-derived M1- and M2-like MΦs were pre-treated with 100 ng/ml K12-LPS for (1: 24h). The final density of cells was  $1 \times 10^6$  cells/ml plated out in 12 well flat-bottomed tissue culture plates. Before re-stimulation with 100 ng/ml K12-LPS the medium was removed carefully and the cells were washed in fresh R10. Afterwards the cells were incubated with 100 ng/ml K12-LPS for 18h at  $37^{\circ}\text{C}/5\% \text{CO}_2$ . Negative controls for tolerisation and stimulation were included in the experiment. The cells were harvested for either cell lysis or RNA extraction and supernatant were separated and stored at  $-20^{\circ}\text{C}$  until required for a cytokine assay by sandwich ELISA (supernatants) where cell lysis was for detection of gene expression by RT-PCR or Western blot (WB).

#### **2.2.3.3 Epithelial cell stimulation**

Caco2 cells were treated with PAMPs (100 ng/ml K12-LPS) and pro-inflammatory cytokines (10 ng/ml  $\text{TNF}\alpha$ , or 5 ng/ml  $\text{IL-1}\beta$ ) and cultured for 24h. Cell supernatants were collected and kept at  $-20^{\circ}\text{C}$  until required for evaluation by ELISA and the cell lysate for of gene expression analysis by (RT-PCR).

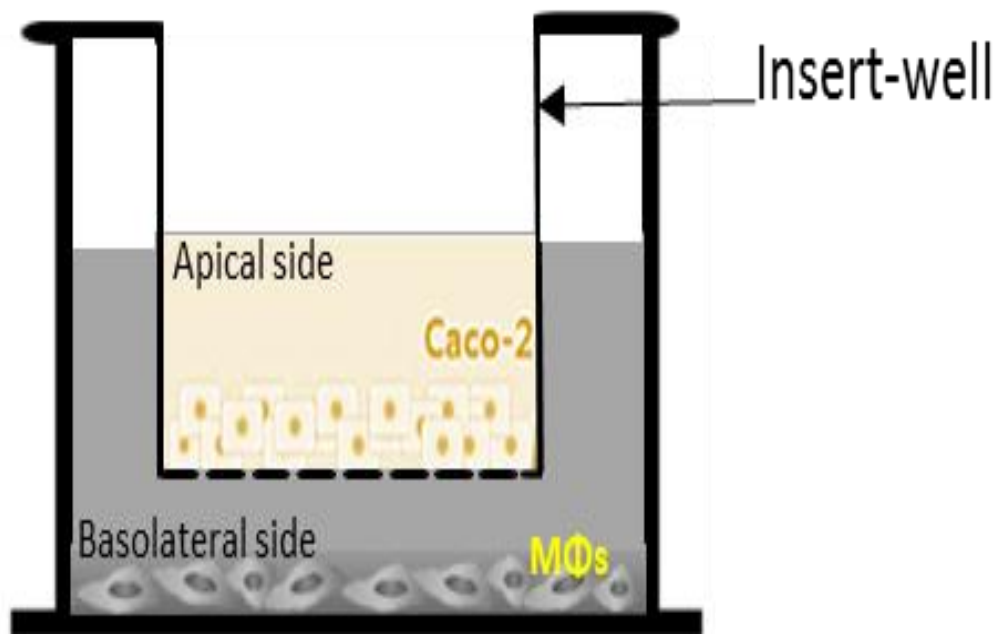
#### **2.2.3.4 $\text{TNF}\alpha$ modulation on LPS-induced MΦ cytokines; (chronic inflammation)**

A concentration of 10 ng/ml of  $\text{TNF}\alpha$  was used to treat MΦ subsets using the pulse chase method for detecting  $\text{TNF}\alpha$  cytokine release by MΦs. The pulse chase method

was performed for detection TNF $\alpha$  cytokine release. The pulse chase method was done by stimulating the cells with TNF $\alpha$  for different time points (0, 1, 2, 4, 6, 9, 12, 18, 24, 48, 72 and 96 h) followed by washing the cells with fresh medium and incubating them with or without LPS for 18h. For detecting other cytokines such as IL-6, IL-18 and IL-10, cells were treated with TNF $\alpha$  (0, 1, 2, 4, 6, 9, 12, 18, 24, 48, 72 and 96 h) followed by washing the cells with fresh medium and incubating them with or without LPS for 18h. The cell supernatants were collected after 18h LPS stimulation and kept at -20°C until used for cytokine detection by ELISA.

#### **2.2.3.5 Epithelial cell- macrophage co-culture system**

A co-culture model was created to mimic the inflammatory gut pathology (homeostatic and inflammatory) where epithelial cells cross-talked with macrophage subsets in the presence of LPS, TNF  $\alpha$  or IL-1 $\beta$ . The co-culture system was made using Caco2 human epithelial cells and THP-1 monocyte-derived macrophage cell subsets M1- or M2-like macrophages (Watanabe et al., 2004a) . Briefly, three groups of Caco2 cells were seeded on 12 trans well cell culture insert (greiner bio-one) plates (0.4  $\mu$ m pore size) at a cell density of  $5 \times 10^5$  cells/500 $\mu$ l/well and cultured in D10 medium in a humidified 5% CO<sub>2</sub> incubator at 37 °C for 21 days allowing for full cell differentiation. Two groups of Caco2 cells grown in transwell inserts were incubated with M1- or M2-like M $\Phi$ s for 24h in the presence of TNF $\alpha$  or IL-1 $\beta$  apically in the co-culture system followed by basolateral stimulation with 100 ng/ml K12-LPS, for 18h. The third group of Caco2 cells was left without co-culture as a control as well as M $\Phi$  subsets controls. The apical and basolateral cell supernatant were collected and stored at -20 °C for analysing by ELISA. Trans epithelial electrical resistance (TEER) assay to test barrier function was determined for each treatment group (Figure 2.2-1).



**Figure 2.2-1: Experimental setup of the co-culture comprising of intestinal epithelial cell line (Caco2) and either macrophage subsets (M1-like or M2-like MΦs).**

For mimicking gut mucosal tissue, co-culture model of Caco-2 cells which were cultured in transwell insert, with THP-1 derived M1-like MΦs (inflammatory model) or M2-like MΦs (homeostatic model), apically treated with LPS, TNF $\alpha$  or IL-1 $\beta$ , and stimulated with LPS in basolateral side depending on designed experiment.

#### **2.2.4 Trans epithelial electrical resistance (TEER) assay**

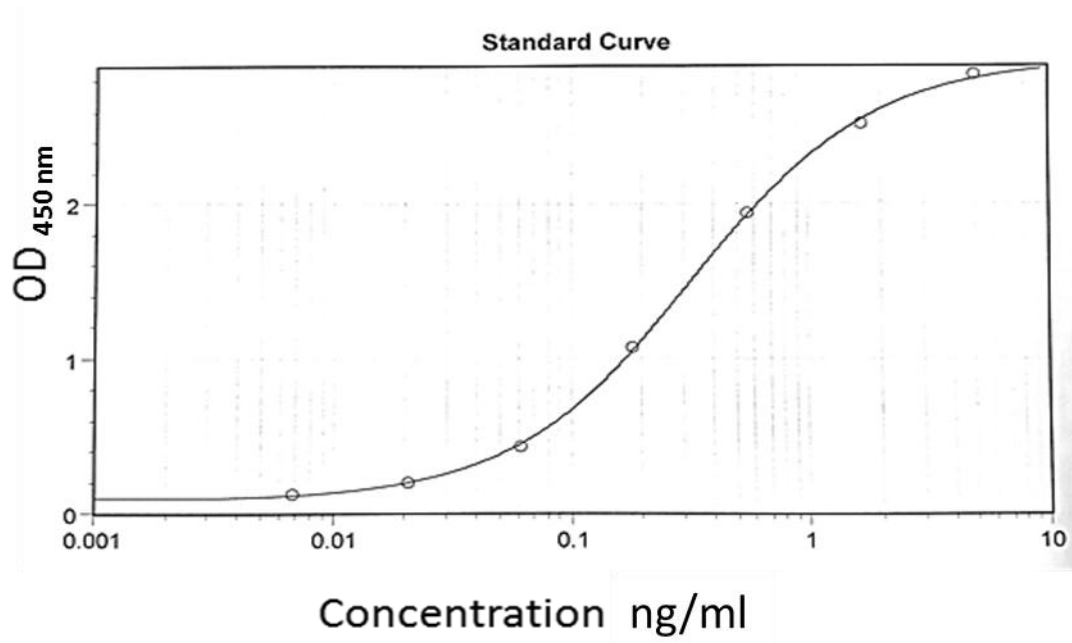
Transepithelial electrical resistance (TEER) measurement was used routinely to study the paracellular transport properties (permeability) of epithelial cells grown on permeable filters and barrier integrity. TEER measurement was done according to the modified protocol by Teoh et al (2000). Caco2 cells were cultured in transwell inserts for 21 days, incubated either alone or with M1- or with M2-like MΦs. During the differentiation time and after the treatment, cells were washed twice with DPBS and 0.5 ml of the DPBS was added to the inner and 1 ml to the outer of the transwell. The electrical resistance was calculated using EVOM Epithelial Voltammeter (Pharma, UK),

where each well reading multiplied by the surface area of the transwell ( $0.33 \text{ cm}^2$ ) to calculate the final value in  $\Omega\text{cm}^2$ .

### 2.2.5 Cytokine measurement

The production of pro-inflammatory cytokines (TNF $\alpha$ , IL-1 $\beta$ , IL-18 and IL-6) and anti-inflammatory cytokines (IL-10) were measured by using sandwich ELISA. The 96 immuno-absorbance well plates were coated with commercially available capture antibodies. Anti-TNF $\alpha$  (0.5  $\mu\text{g/ml}$ ), anti-IL-1 $\beta$  (1  $\mu\text{g/ml}$ ), anti-IL-18 (0.5  $\mu\text{g/ml}$ ), anti-IL-6 (1  $\mu\text{g/ml}$ ), anti-IL-8 (2  $\mu\text{g/ml}$ ), anti-IL-10 (0.5  $\mu\text{g/ml}$ ) and incubated overnight at 4  $^{\circ}\text{C}$ . The plates were washed twice with washing buffer before being blocked with 2%w/v BSA/PBS for 4h at room temperature (RT). Then, plates were washed three times by washing buffer and incubated with serially diluted recombinant cytokine standards and test samples and incubated at 4  $^{\circ}\text{C}$  overnight. Next day, plates were washed three times by washing buffer followed by incubation with biotinylated anti-TNF $\alpha$  (0.5  $\mu\text{g/ml}$ ), anti-IL-1 $\beta$  (100 ng/ml), anti-IL-18 (1  $\mu\text{g/ml}$ ), anti-IL-6 (10 ng/ml), anti-IL-8 (5 ng/ml), and anti-IL-10 (1  $\mu\text{g/ml}$ ) for 4h at RT. Plates were washed three times with washing buffer and incubated with 50ul/well biotinylated streptavidin horseradish peroxidase (HRP) at 1/250 dilution in 2%w/v BSA/PBS for 1 h at RT. Finally, the plates were washed three times with washing buffer followed by the addition of colour reagent (Tetramethylbenzidine-TMB, Middlesex UK) until the colour is developed at RT. The reaction was stopped by adding 100  $\mu\text{l}$  of 1.8 M sulphuric acid to each well. Colorimetric development was determined spectrophotometrically by the OPTIMax tuneable microplate reader at 450nm and analysed by Softmax Pro version 2.4.1 software (Molecular Devices Corp., Sunnyvale, CA, USA). Protocols are applied according to manufacturer's instruction. Standard curves, between ranges (7 to 5000

pg/ml), were used to determine the cytokine concentration and compared with the recognized international standards available from (NIBSC, Potter's Bar, UK). Reagents and Antibodies used for ELISA are available in appendix 1, Table 2-1.



**Figure 2.2-2: Standard calibration curve of ELISA.**

Standard curves, between ranges (7 to 5000 pg/ml), were used to determine the cytokine concentration and compared with the recognized international standards available from (NIBSC, Potter's Bar, UK). The absorbance (OD) was determined in a micro-plate reader at 450 nm. Data displayed is a representative experiment with triplicate samples of n= 3 replicate experiments.

## 2.2.6 Detection of IL-10 membrane bound protein by macrophage subsets

Endogenous cell-associated IL-10 was examined depending on anti-inflammatory activity of IL-10 to down-regulate LPS-induced TNF $\alpha$  (Fleming and Campbell, 1996). M1- and M2-like M $\Phi$ s were incubated with 10 mg/ml 9D7 neutralising anti-IL-10 antibody obtained from Biolegend (San Diego, USA) or irrelevant, isotype-matched control antibody for 30 min then stimulated with 100 ng/ml K12-LPS and cultured for 18h at 37  $^{\circ}$ C, 5% CO $_2$ . Furthermore, M $\Phi$  sensitivity to exogenous IL-10 was evaluated

by pre-treating M1 and M2 MΦs with exogenous IL-10 (0, 0.2, 2 and 20 ng/ml) (NIBSC, Potter's Bar, UK) for 30 min prior to challenge with 100 ng/ml K12-LPS for 18h. Supernatants were harvested for TNFα secretion valuation by sandwich ELISA. MΦ viability was routinely checked by Trypan blue exclusion (viability routinely >85%).

### **2.2.7 Role of caspase-1 in macrophages cell signaling**

The role of Caspase-1 in macrophages cell signaling was evaluated by using Caspase-1 Inhibitor Z-WEHD-FMK obtained from (R&D SYSTEM, Abingdon UK). THP-1 derived M1- and M2-like MΦs were pre-treated with range of concentration of Caspase-1 Inhibitor (0 μM, 10 μM, 20 μM and 50 μM) for 1 h prior to stimulation with 100 ng/ml K12-LPS for a further 18h incubation at 37 °C, 5% CO<sub>2</sub>. Cell viability was checked by Trypan blue exclusion and supernatant was collected and stored at -20 °C for human IL-1β and IL-18 ELISA.

### **2.2.8 Protein analysis methods**

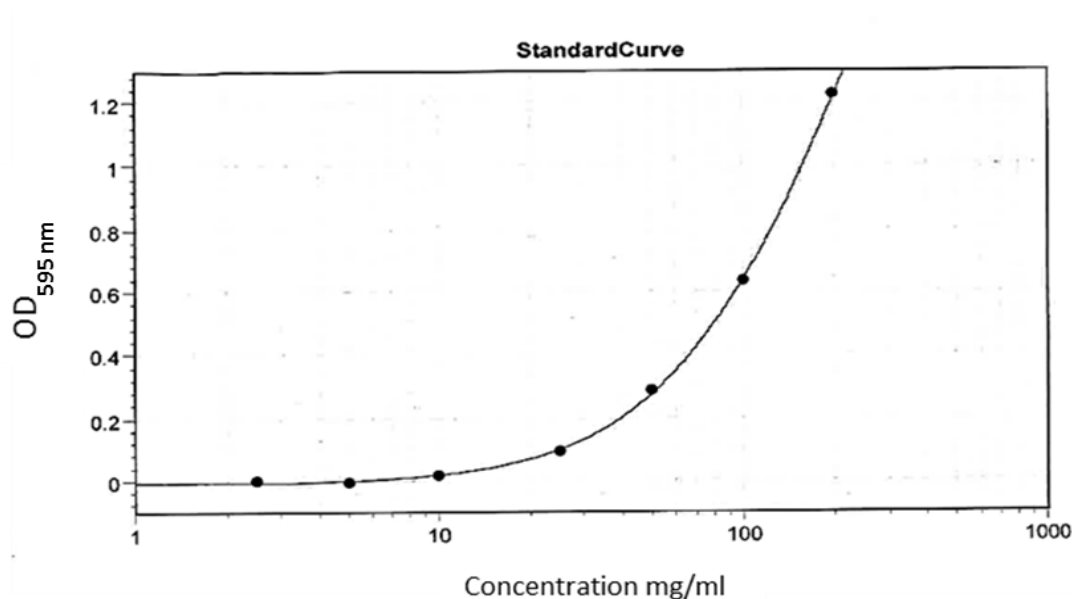
#### **2.2.8.1 Cell lysis**

After specific treatments, M1- and M2-like Macrophage cells were washed gently with PBS followed by adding lysis buffer, RIPA buffer supplemented with a protease/phosphatase inhibitor cocktail. Then, cell lysate was centrifuged at 3,500 rpm at 4 °C for 10 minutes to spin down cell remains into a pellet. Supernatants were carefully separated without disturbing the pellet and stored at -20 °C for protein concentration to be determined by the Bradford method prior WB (refer appendix 1Table 3-1).

#### **2.2.8.2 Protein quantification**

The method that was used to quantify the protein of interest was the Bradford method (Bradford, 1976) modified for the micro-plate reader. This method is suggested for

common use, particularly detection of protein of cell segments and secreted extractions (Ernst and Zor, 2010). This assay aims on the determination the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when involvement with protein existence (Bradford, 1976). Bovine Serum Albumin (BSA) protein was used as standards between 7  $\mu\text{g/ml}$  and 5 mg/ml which were prepared by dissolving BSA in distilled water followed by adding an equal volume of dye reagent (Bradford) then incubating for 5 min at RT. The absorbance was measured in a micro-plate reader (Molecular Devices, Manchester, UK) at 595 nm where standard curve was shaped between absorbance value and protein concentrations, and the experiment samples were calculated according to extrapolation from the standard curve (Figure 2.2-3).



**Figure 2.2-3: Standard calibration curve of BSA by Bradford assay**

BSA's standard curve of Bovine serum albumin (BSA) protein [7  $\mu\text{g/ml}$ : 5 mg/ml]. The absorbance (OD) was determined in a micro-plate reader at 595 nm. Data displayed is a representative experiment with triplicate samples of  $n=3$  replicate experiments.



### **2.2.8.3 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE is an analytical technique for recognition complex mixtures of proteins. This method was applied using a Laemmli discontinuous buffer system. To prepare SDS gel, glass plates (BIO-RAD, UK) were cleaned with detergent and washed with deionised water, followed by 70% v/v ethanol then left to dry. The final concentration of the determining gel was 12.5% w/v. The required gel solutions were firstly mixed with fresh 1% w/v ammonium per sulphate (APS) and Tetramethylethylene diamine (TEMED) (see appendix 1 Table 3-1) for polymerisation and immediately loaded into the preformed space between the glass plates and filled to two thirds the height of the largest glass plate. Two ml of 10% v/v water saturated isopropanol was loaded immediately on top of the deciding gel making a layer on top of the gel to release the air bubbles. The gel was kept for polymerisation for 30 min at RT. Then, the isopropanol layer was detached and the stacking gel 3%w/v was prepared depending on the recipe mentioned in followed by adding APS and TEMED, and then immediately loaded on top of the prepared gel. Then to make wells for sample's loading, a comb was inserted into the stacking gel and left the gel for few min for polymerisation and transferred it to the electrophoresis container. The container was filled with running buffer covering the top of the gel. The comb was then carefully removed and the wells were washed with running buffer. Twelve  $\mu$ l of each protein sample and 3  $\mu$ l of loading buffer were boiled for 3 min to denature the protein, quenched on ice for 2 min, briefly vortexed, centrifuged at 12000 rpm for 30 sec then loaded into each well of the gel. The gel was run at 100 V for 2 h and the dye marker was observed (Hyper Page pre-stained protein Marker, Bio line, UK). The gel was removed from the

electrophoresis system container, plates were removed, and the gel was ready for transfer to PVDF blotting membrane.

#### **2.2.8.4 Western blotting (WB)**

Western blotting is commonly applied for identifying specific proteins stained with specific antibodies in a specified complex sample protein. Proteins of interest in this investigation include: TLR4, IRAK-M, Tollip, SIGIRR, Phospho-STAT3, total STAT3, and GAPDH (refer appendix 1, Table 3-1 for more information). Proteins are detected by SDS-PAGE or Criterion Xt precast gel (Bio-Rad Laboratories Ltd) then proteins were transferred to polyvinylidene fluoride (PVDF) membranes by using an electro-blotter system (Criterion blotter, BIO-RAD, UK), which were cut to the same size as the gel, along with two pieces of filter papers. In order to prepare PVDF, it was soaked in methanol (100%v/v) for 15s then distilled water for 2 min then in transfer buffer for 5 min. The blotting device system was gathered as follows: black plastic cassette plate at the bottom, gauze, filter papers, gel, PVDF blotting paper, filter papers, gauze, and red plastic cassette at the top. The cassette was firmly closed prior to removing the air bubbles with a roller, and then the cassette was inserted in the tank where the red side towards the cathode (red electrode). Cold transfer buffer was added to the tank, an ice pack was placed at the bottom as well as a magnetic stirrer for cooling supply, and to avoid the overheating of the buffer, which might affect the blotting system.

Blotting system was connected to the power and the gel was electro blotted at 100 V for 35-45 min. When blotting is complete, the PVDF membrane was blocked with blocking (refer to appendix table 3-1) solution at RT for 1h on the roller followed by incubation the membranes overnight with the specific interest primary antibody in 1% BSA in TBST at 4 °C on the roller. Next day, primary antibody was placed into an empty

falcon tube and stored at -20 °C (can be used twice), and PVDF membrane was washed with TBST three times, 5 min each at RT on the roller. Then, the membranes were incubated for 2h at RT with suitable secondary antibodies together with horseradish peroxidase (HRP). The membranes were washed three times for 5 min with TBST at RT on roller followed by washing once for 5 min with TBS on roller to remove traces of Tween-20. To detect peroxidase activity from HRP-conjugated antibody, developing colour for protein detection was achieved by using Luminata Crescendo Western HRP substrate, 100ml (Millipore Corp/ Merck Group, Hertfordshire UK). After washing with TBS, the membrane was removed and laid out on a plastic sheet. Developing solution was carefully loaded over the entire membrane for few min. The protein bands were pictured by a gel documentation system using EC3 imaging system (UVP ultra violet product, Ca, UK). Band densities were quantitated by ImageJ 1.47t software then the ratio of optical density compared to the negative control that was normalized to GAPDH controls.

## **2.2.9 Gene expression analysis methods**

(Refer to appendix 1 Table 4-4-1 for material and reagents)

### **2.2.9.1 Total RNA extraction**

Total RNA was extracted using a Sigma GenElute mammalian total RNA extraction kit following the manufacturer's procedure for each treatment of the cells. Adherent M1-macrophages were washed twice with PBS, then adherent cells were harvested by a sterile rubber cell scraper (Sterlin, UK), whereas, M2 cells were collected by centrifugation at 1200 rpm for 5min. After washing step, the cells were re-suspended in 500 µl of lysis buffer provided by the kit, supplemented with 5 µl 0.02% v/v 2-mercaptoethanol for denaturation of RNase (reducing disulphide bonds and destroying

the enzyme functionality of RNases released during the lysis step of RNA isolation). DNase digestion with RNase-Free DNase was used according to the protocol, to remove potential DNA contamination. This was done by adding 10 µl of DNase and 70 µl of digestion buffer on the top of each filter of the column used for RNA isolation and the columns were incubated for 15 min at RT. Extracted RNA from each sample was eluted in 50 µl of elution buffer provided with RNA extraction kit.

#### **2.2.9.2 RNA quantification and evaluation**

RNA quantification was done by using spectrophotometer, Nanodrop measuring both the concentration of RNA and purity (contamination of proteins and DNA). The ratio of OD260/OD280 has to be between 1.8 and 2.0 to exclude any contamination in the RNA sample extracted (Liu et al., 2003). Purified RNA was immediately stored at -20 °C until required for gene expression analysis. The integrity of RNA sample was checked by running 1% formaldehyde agarose gel according to the protocol of Van Dessel et al 2004. In brief, the formaldehyde agarose gel was prepared by mixing 87.5 ml distilled sterile water. 1.5 g agarose, 10 ml 10xMOPS prepared in flask and heated in microwave for 2 min to dissolve all agarose. Subsequently, 7µl of Syber safe or red gel was added into small gel set followed by shaking to mix all the components of agarose liquid then cooled to 60 °C prior to adding 26 µl formaldehyde with stirring in the fume hood. The mixture was poured out into the BIO-RAD gel set and left for casting. The gel was mounted in an electrophoresis tank and overlaid with 1xMOPS electrophoresis buffer. For loading buffer, the mixture was prepared as follows (2µl 1-x MOPS, 3.5 µl formaldehyde, 10 µl formamide, 0.4%w/v Bromophenol blue) a volume of 15.5µl of loading buffer and 4.5µl of sample were mixed together and incubated for 10 min at 65 °C to control RNase contamination and denaturation, quenched on ice for 10 min,

followed by brief centrifugation and loaded into the gel well. The gels were run at 40-60 volts for 60 min, de-stained by using deionised water for 45 min, and reviewed under UV light using the documentary gel reviewer (Gel Doc™.XR, BIO-RAD, CA).

#### **2.2.9.3 Reverse transcription of total RNA**

Total RNA was reversed transcribed to complementary DNA (cDNA) by M-MLV Reverse transcription reaction Kit. Following a modified manufacture's protocol, 10µl containing 1µg/ml RNA of sample and 1µl of 10mM dNTP mixture, 1µl of random nanomer topped up with nuclease free water to make cDNA from a template of 1 µg/ml total RNA. The mixture was incubated at 70 °C for 10 min, after that the mixture was placed on ice for at least 2 min. A total of 10 µl containing 1ul M-MLV enzyme, 2µl of buffer, 0.5 µl of RNAase inhibitor and 6.5 µl of nuclease free water was added to each sample. The cDNA synthesis was achieved after incubation at 37 °C for 50 min where reaction was terminated immediately by heat inactivation for 10 min at 95 °C before chilling at 4 °C.

#### **2.2.9.4 Design of primers**

The full length sequences of the gene of interest were identified using the National Centre of Biotechnology Institute (NCBI). Primer express software provided with step one PCR machine (Applied Biosystems, Lingly House, Warrington, UK), was used to design the primer based on the sequence number of the nucleotides, and melting temperature. The following setting were used for primer design ( Primer T<sub>m</sub>: 60-65 °C, primer length: 20-28 bp, Amplicon size: 100-160 bp) to ensure that the primers were designed match the same gene map sequenced, the primers were plasted by using NCBI blast software at <http://blast.ncbi.nlm.nih.gov/>, and the primers characteristics were assessed using Sigma DNA calculator website <http://www.sigma->

[genosys.com/calc/DNACalc.asp](http://genosys.com/calc/DNACalc.asp). The primers were synthesised by Eurofin MWG/ Operon (Germany). The lyophilized primers were dissolved in appropriate volumes of nuclease free water to prepare 10 pmol of each primer depended on MWG instruction and then stored at -20 °C. In this study the expression of TNF $\alpha$ , IL-6, IL-1 $\beta$ , IL-10, TLR2, TLR4, NOD-2, Tollip, IRAK-M and SIGIRR gene expression were quantified using RT-PCR (refer to appendix 1 Table 4-4-2 for primers details) .

#### **2.2.9.5 Real Time PCR**

RT-PCR assay relies on the assumption that the housekeeping gene levels of expression remain constant in different cells, samples, and treatments. Consequently, housekeeping gene have been used to normalise the data. According to a modified manufacturer's protocol (Applied Bio systems, Warrington, Cheshire, UK), a total volume of 12  $\mu$ l composed of 1  $\mu$ l cDNA of each sample, 0.25  $\mu$ l of 10 pmol of specific primers for each target (forward and reverse), 3.5  $\mu$ l of Power SYBER Green® (Applied Biosystem, UK) and 7.0  $\mu$ l of nuclease free water was plated out into 96-well plates (Applied Bio system, UK), and then sealed with adhesive film. RT-PCR was applied using Step One Plus thermal cycler whereby the amplification of target was carried out under the following conditions; pre-heating at 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 30 seconds, 60 °C for 1 minute and 72 °C for 1 minute and hold the samples at 4 °C. RT-PCR data were analysed following  $2^{-\Delta\Delta C_t}$  method as described in literature, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control and resting cells as a reference sample (Livak and Schmittgen, 2001). Therefore, the comparative quantity of the target transcript is defined as fold change relative to the reference sample (resting cells) and GAPDH using the following equation:

$$\Delta\Delta C t = \Delta C t \text{ interest gene (resting cells)} - \Delta C t \text{ endogenous control}$$

$$\text{Relative quantitation (RQ)} = 2^{-\{\Delta\Delta C T \text{ sample} - \Delta\Delta C t \text{ control (resting cells)}\}}$$

Where CT is threshold cycle

### 2.2.10 Statistical analysis

Measure of statistical significance was analysed using a balanced analysis of variance (General Linear Model, Minitab version 16). All data were tested for normality using the Anderson-Darling test (with  $P < 0.05$  indicating that the data were non-normal). Levene's test was used to check that there were no significant differences in variance between each group ( $P < 0.05$  indicated a significant difference in variance between groups). For normally distributed data, significant differences were determined using one way ANOVA followed by Bonferroni tests as *post hoc* multiple comparison tests for normal data, a multiple comparison test (ns= no significant difference). Significance was set at p values; (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ).

**Chapter 3: Validation of THP-1  
derived MΦs as a model of M1- &  
M2-like MΦs in homeostatic and  
inflammatory environments.**



### 3.1 Introduction

Macrophages (MΦs) are phagocytic cells of the innate immune system, which occur in most tissues of the human body. These cells reveal a wide selection of functional features including phagocytic clearance, microbial killing, antigen processing and presentation, inflammation, anti-inflammatory processes, tissue repair and immune suppression. This variety of immune functionality is revealed in macrophage subset heterogeneity. Present finding classify MΦ subsets according to activation status (classical or alternative) or differentiation (M1 or M2) reviewed in (Foey, 2012). Mucosal macrophages have an essential part in tolerance; while in inflammatory bowel disease, (IBD) dysfunctional macrophages initiate the breakdown of tolerance and commensals perpetuate inflammation. Gut macrophages control immune homeostasis, driving tolerogenic reactions to food and commensal bacteria or immune inflammatory responses to pathogens (Smith et al., 2011). In the gut environment, tolerance regulation is associated with an M2-like phenotype, whereas inflammatory activation is associated with an M1-like phenotype (Gordon and Taylor, 2005). M2-like macrophage is the subset that predominates in a healthy gut mucosa. Disruption of tissue homeostasis by pathogenic microbial infection leads to macrophage activation, which is related with macrophage phenotype alterations from M2s to M1s depending on the signals received from the tissue environment (Platt and Mowat, 2008).

Human THP-1 cells were used as a macrophage model in the current study. It has been reported that when THP-1 cells were treated with PMA, the cells differentiated into macrophage-like cells, which mimic native monocyte-derived macrophages in many respects (Auwerx, 1991, Schwende et al., 1996). This delivers a valued model for

macrophage differentiation and regulation studying. Several studies have used this method of THP-1 cells line differentiation by PMA or Vit.D<sub>3</sub> to characterise macrophage subsets see (Table 3.1-1).

It has been established, according to modification in markers expression, that the THP-1 cell line can be differentiated to two distinct macrophage subsets; M1-like and M2-like, upon treatment with PMA or Vit.D<sub>3</sub> respectively. The present study aimed to determine the effect of PAMPs and pro-inflammatory cytokine TNF $\alpha$  and IL-1 $\beta$  treatments on modulation of the immune responses of these macrophage subsets. Then lipid A flows into the blood, binds to the immune cell surface through contact with TLR4/MD-2/CD14 and triggers the immune responses associated with fever, diarrhoea, and septic shock. Certainly, activation of the NF- $\kappa$ B transcription factor is a result of LPS stimulation of M $\Phi$ s, which orchestrates a gene expression schedule resulting on inflammation, cell proliferation (Th1), differentiation (Th17), through the release of chemokines and cytokines.

The pro-inflammatory cytokine TNF $\alpha$ , produced by monocytes and macrophages directly after recognition of pathogen, has an important role in innate immunity, mutually directly and indirectly by increase of TLR-induced production of additional pro-inflammatory cytokines such as IL-1 $\beta$ , IL-18, IL-12, IL-6, and IL-8 (Beutler, 1999). Uncontrolled secretion of TNF $\alpha$  lead to development and progression of chronic inflammation and autoimmune diseases such as IBD, rheumatoid arthritis (RA) and psoriasis (Williams et al., 2007).

Another important cytokine that plays a crucial role in many inflammatory conditions of the gut immune system is IL- $\beta$ . IL-1 $\beta$  has a role in both the initiation and amplification of the inflammatory response, which result in intestinal injury. IL-1 $\beta$  also

is involved in the pathogenesis of intestinal inflammation in IBD and animal models of intestinal inflammation. In the case of imbalance between IL-1 $\beta$  and its antagonist, IL-1ra exists in the intestinal mucosa of IBD patients. This imbalance proposes that the absence of anti-inflammatory role of IL-1 to respond the raised levels of IL-1 could be a significant pathogenic fault (Al-Sadi and Ma, 2007).

Inflammasomes are key signaling machines of the innate immune system that drive the production of the highly inflammatory cytokine IL-1 $\beta$  via caspase-1 activation in response to microbial and nonmicrobial danger signals (Schroder and Tschopp, 2010). Some individual inflammasomes have been identified; each differentiated by specific motivators, NLR/ALR family members, and caspase effectors. The typical or recognised inflammasome complex contains of a cytosolic sensor (a nucleotide-binding domain and leucine-rich-repeat-containing [NLR] protein or an AIM2-like receptor [ALR] protein), an adaptor protein (apoptosis-associated speck-like protein containing a CARD [ASC]), and an effector caspase pro-caspase-1 (von Moltke et al., 2013). ASC is a divided molecule that comprises mutually an N-terminal Pyrin domain (PYD) and a C-terminal caspase initiation and recruitment domain (CARD), allowing it to link the receptor (NLRs or ALRs) and the effector pro-caspase-1 (Rathinam and Fitzgerald, 2016). The Inflammasome Part Pycard Is Essential for the Activation of Caspase-1 and Caspase-5. It has been demonstrated that caspase initiation was observed in lysates in the presence of two different anti-Pycard antibodies (a polyclonal [ $\alpha$ -Pycard] or a monoclonal antibody [ $\alpha$ -ASC]), activation of both caspase-1 and caspase-5 (Martinon et al., 2002). Caspase-1 in turn regulates the proteolytic maturation of interleukin-1 $\beta$  and IL-18, as well as a rapid, noxious, inflammatory form of cell death termed pyroptosis (Rathinam et al., 2012a).

**Table 3.1-1: Validation markers involved in THP-1 derived macrophage subsets**

Marker	M1 (+PMA)	M2 (+Vit.D <sub>3</sub> )	Reference
LPS-stimulated TNFα (protein)	↑	↓	(Schwende et al., 1996) (Daigneault et al., 2010)
Morphological changes	pseudopodia	ruffled	
TNFα gene expression	↑	↓	(Chanput et al., 2010)
LPS-induced IL-12	↓	↑	(Gynther et al., 2011)
LPS-stimulated IL-10	↓	↑	(Matilainen et al., 2010)
LPS stimulated IL-β and IL-8 (mRNA)	↑	↓	(Chanput et al., 2010)
IL-8 gene expression	↑	↓	
TLR expression	TLR2 (high) TLR4 (high)	TLR10 (high)	(Verma et al., 2014)
iNOs (mRNA)	↑	ND	(Chanput et al., 2010)
Arginase (mRNA)	ND	↑	
MRC-1, Dectin-1 (mRNA)	↓	↑	(Chanput et al., 2010)
IL-12p40 (mRNA)	↑	↓	
MRC-1 gene expression	↓	↑	(Chanput et al., 2013)
DC-SIGN	↓	↑	
CD36, PPAR-γ, CD204, PKC delta isoform	↑	ND	(Barilli et al., 2011)
CD206 (mRNA) expression	↓	↑	(Daigneault et al., 2010)
Phagocytic capacity	High phagocytic capacity	↑ more than in M1	
ND = not detected, ↑ increase, ↓ decrease			

In this chapter, the investigations focused on determining the influence of *E.coli* K12-LPS and PAMPs on modulation of MΦ subset behaviour via cytokine production. This was evaluated in MΦ subsets derived from THP-1 human monocytic cell line resembling either infiltrating pro-inflammatory MΦ, M1s, or mucosal resident homeostatic MΦ, M2s. In addition, the roles of pro-inflammatory cytokines, TNFα challenge, in long and short term, in modulation of K12-LPS-induced MΦs was investigated. Finally, inflammasome activity in MΦ subsets has been studied in this chapter concerning IL-1β and IL-18 production, role of caspase-1 as well as IL-β challenge.

The particular hypotheses to be verified in this investigation are:

**Hypothesis 1:** THP-1 derived MΦs; M1- and M2-like, differentially respond to different PAMPs include TLR4 agonist; *E. coli* LPS, TLR2 agonist PG-LPS, and LTA and NOD1/2 agonist PGN.

**Hypothesis 2:** pro-inflammatory cytokines, TNFα and IL-1β, can modulate K12-LPS induced cytokine production in macrophage subsets.

**Hypothesis 3:** *E. coli* LPS is able to modulate inflammasome activation in macrophage subsets.

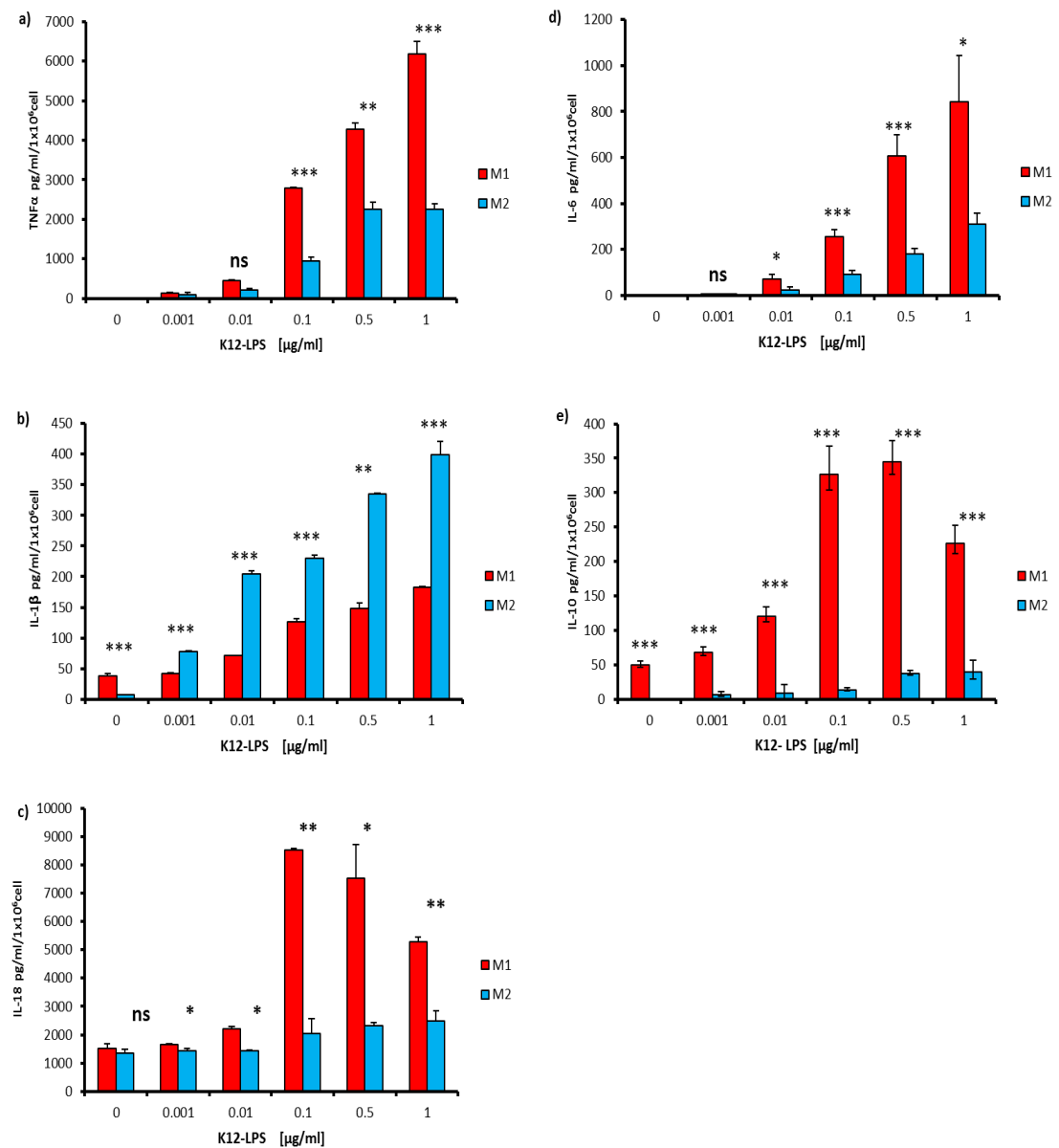
## 3.2 Results

### 3.2.1 PAMPs modulate M1- & M2-like MΦs effector function.

#### 3.2.1.1 M1- & M2-like MΦs stimulated by K12-LPS induce separate pro- and anti-inflammatory cytokine profiles.

In order to obtain full cytokine production profile by macrophage subsets and to find the right concentration of K12-LPS, this experiment was designed. Macrophage subsets differentially respond to different concentrations of *E. coli* LPS. Challenge these MΦs was comparable between the two subsets and according to the concentrations. Overall, when stimulated by K12-LPS, pro-inflammatory M1 MΦs expressed higher levels of TNFα, IL-18, IL-6 and IL-10 cytokines compared to anti-inflammatory M2-like MΦs, which interestingly produced higher level of IL-1β (Figure 3.2-1). The production level for TNFα was low with no significant between M1 and M2 when stimulated with 0.01 µg/ml K12-LPS or less. TNFα induced by M1-like MΦs was significantly higher than TNFα induced by M2-like MΦs when stimulated by 0.1 0.5 and 1 µg/ml K12-LPS and P= 0.001 with all concentrations. The 50% effective dose (ED<sub>50</sub>) of K12-LPS was 0.482 µg/ml and 0.45 µg/ml for M1- and M2-like MΦs, respectively. On the other hand, IL-1β was induced significantly in higher levels by M2-like MΦs by all used concentrations and the concentration of IL-1β increased gradually in parallel with stimulus concentration by both MΦ subsets. The P < 0.001 in all concentrations of LPS except 0.5 µg/ml where P= 0.006. Remarkably, M1-like MΦs produced a certain amount of IL-1β in absence of K12-LPS, which might be in response to PMA. Remarkably, ED<sub>50</sub> of K12-LPS was 0.25 µg/ml for both M1- and M2-like MΦs. The level of pro-inflammatory cytokine, IL-18, was slightly higher by M1-like with low LPS concentrations, 0.001 and

0.01 µg/ml and the  $P \leq 0.05$  for both concentrations. IL-18 was produced significantly in high level by M1-like, with higher concentrations; 0.1 µg/ml  $P = 0.008$ , 0.5 µg/ml  $P = 0.02$  and 1 µg/ml  $P = 0.009$ . However,  $ED_{50}$  of K12-LPS was 0.03 µg/ml and 0.35 µg/ml for M1- and M2-like MΦs, respectively. IL-6 level was significantly higher by M1 MΦs upon LPS stimulation with most concentrations. The lowest concentration that displayed significant difference in IL-6 level between M1 and M2 MΦs is 0.01 µg/ml  $P = 0.029$ . IL-6 cytokine concentration increased steadily in parallel with LPS dose dependency concentration. Remarkably,  $ED_{50}$  of K12-LPS was 0.41 µg/ml for both M1- and M2-like MΦs. IL-10 level was significantly higher by M1 MΦs upon stimulation with the same concentration. IL-10 production was comparable between M1 and M2 MΦs with all concentrations of K12-LPS with  $P < 0.001$ . However,  $ED_{50}$  of K12-LPS was 0.17 µg/ml and 0.12 µg/ml for M1- and M2-like MΦs, respectively. After challenge with 0.1 µg/ml K12-LPS, M1-like MΦs induced cytokines  $TNF\alpha$ , IL-1 $\beta$ , IL-18, IL-6 and IL-10 at a ratio of 93:4:284:8:11 compare to unstimulated control. On the other hand, M2 MΦs induced  $TNF\alpha$ , IL-1 $\beta$ , IL-18, IL-6 and IL-10 in ratio of 31:8:68:3:0.5 when stimulated by 0.1 µg/ml K12-LPS. The cytokine expression between these two MΦ subsets was significant where  $P = < 0.001$  for  $TNF\alpha$ ,  $P < 0.001$  for IL-1 $\beta$ ,  $P = 0.008$  for IL-18  $P = 0.001$  for IL-6 and  $P = 0.001$  for IL-10 (Figure 3.2-1).



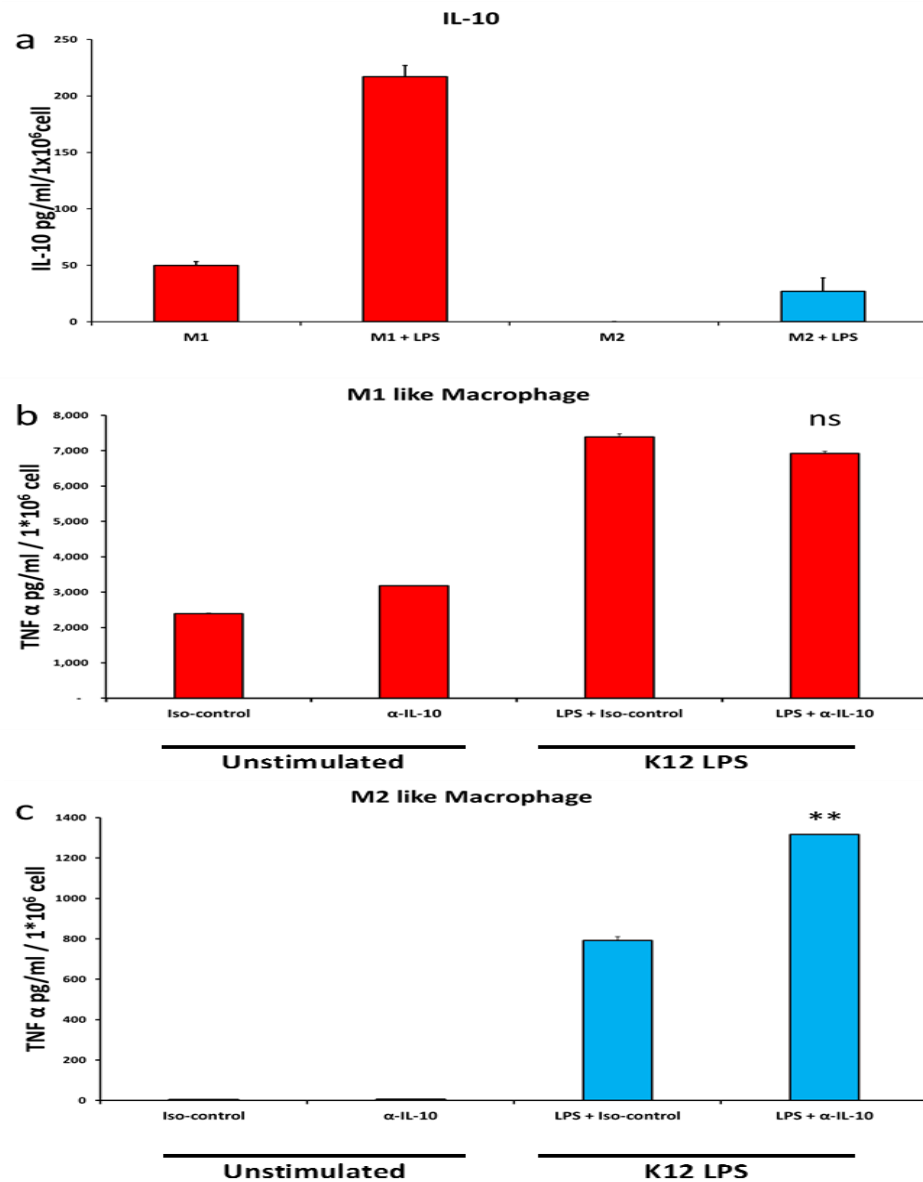
**Figure 3.2-1: K12-LPS stimulated M1 & M2 MΦ subsets exhibited different cytokine profiles.**

THP-1-derived M1 and M2 MΦs were generated by differentiating THP-1 monocytes with either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 3 days or 10 nM 1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> for 7 days, respectively. M1 (Red) and M2 (Blue) MΦ subsets were stimulated in the presence or absence of K12-LPS at concentration titration of [0.001, 0.01, 0.1, 0.5 and 1 μg/ml] for 18h. Cytokine production is expressed as the mean ± SD in pg/ml for TNFα (a), IL-1β (b), IL-18 (c), IL-6 (d) and IL-10 (e). Data displayed represents triplicate samples for n= 3 replicate experiments. Significant differences in cytokine production between K12-LPS activated M1 and M2 MΦs are indicated as \* p < 0.05, \*\* p < 0.01, \*\*\* P < 0.001 and ns= not significant.



### **3.2.1.2 M1 & M2 MΦs display differential IL-10 profiles in response to K12-LPS.**

In general, M1 and M2 MΦs produce different cytokine profiles. M1s show a predominantly pro-inflammatory profile while M2s display a more anti-inflammatory/regulatory profile. This study was undertaken to establish whether M1 and M2 MΦs respond differently with respect to production of IL-10 in response to *E. coli* LPS. Certainly, *E. coli* LPS [100ng/ml] induced individual subset-specific cytokine profiles. Remarkably, K12-LPS induced minimal secretion of IL-10 by M2-like MΦs ( $P=0.014$ , near to lower level of sensitivity of ELISA), compared to M1 MΦs (Figure 3.2-2 a). However, IL-10 production by M1 was measured at high levels ( $217 \pm 10$  pg/ ml,  $p < 0.001$ ). Besides to secretion, bearing in mind membrane bound IL-10 that has been demonstrated (Fleming et al., 1999), this endogenous activity was also studied using neutralising anti-IL-10 antibody in the context of TNF $\alpha$  down-regulation. No endogenous activity was displayed by unstimulated MΦs (Figure 3.2-2 b & c). After K12-LPS stimulation however, MΦ subsets displayed differential production of endogenous IL-10 activity. K12-LPS failed to produce an endogenous suppressive IL-10 activity in M1s; induction of TNF $\alpha$  did not display a statistically significant modification ( $P=0.12$ ) between isotype-matched control and neutralising anti-IL-10 antibody (Figure 3.2-2 b). K12-LPS did, on the other hand, produce an endogenous IL-10 activity expressed by M2-like MΦs. Neutralisation of IL-10 activity increased TNF $\alpha$  production by 40% ( $P < 0.001$ ) (Figure 3.2-2 c).

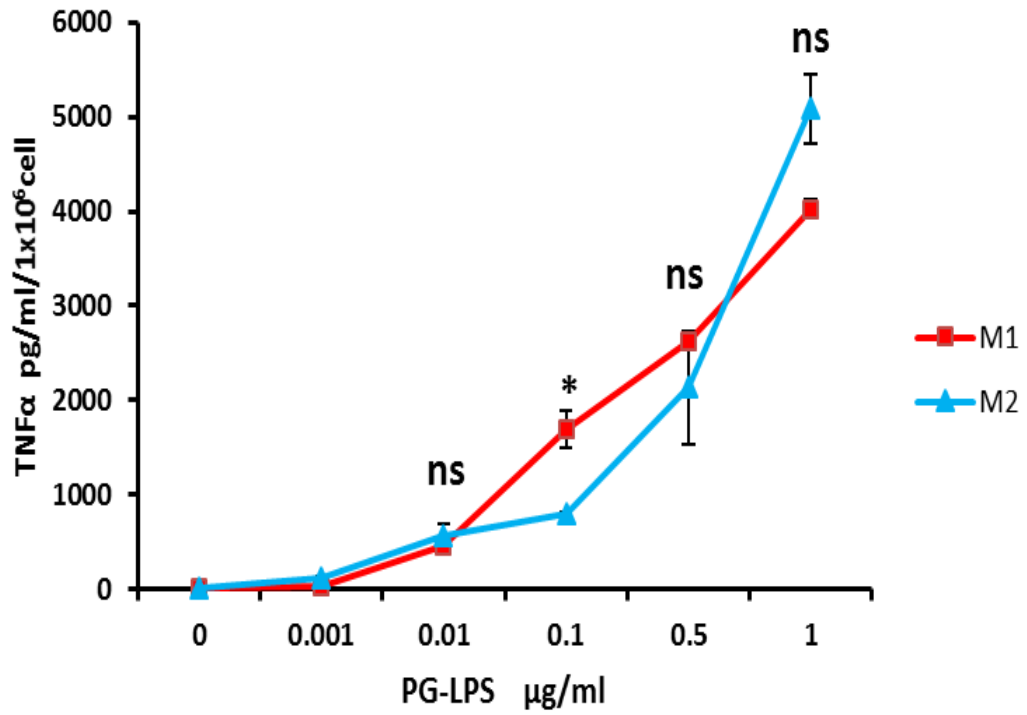


**Figure 3.2-2: M1 & M2 MΦs display differential IL-10 profiles in response to K12-LPS.**

THP-1-derived M1 and M2 MΦs were generated by differentiating THP-1 monocytes with either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 3 days or 10 nM 1, 25-(OH)<sub>2</sub> vitamin D<sub>3</sub> for 7 days, respectively. Secretion of IL-10 (a) is depicted where M1 (red) and M2 (blue) MΦ subsets were stimulated with or without (control) 100 ng/ml K12-LPS. Endogenous IL-10 activity, upon treatment with 10 µg/ml neutralising anti-IL-10 compared to an isotype-matched control antibody (IC), is represented for K12-LPS-stimulated and unstimulated (b) M1-like and (c) M2-like MΦs TNFα secretion. Cytokine secretion is expressed as the mean ± SD in pg/ml for IL-10 (a) and TNFα (b & c). Data displayed represents triplicate samples for n= 3 replicate experiments. Significant differences in cytokine production between activated M1 and M2 MΦs and unstimulated controls and between isotype control and neutralising IL-10 antibody treatment are indicated as \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 and ns, not significant.

### **3.2.1.3 M1- & M2-like MΦs differentially produced TNFα cytokine upon stimulation by the TLR2 agonist, PG-LPS.**

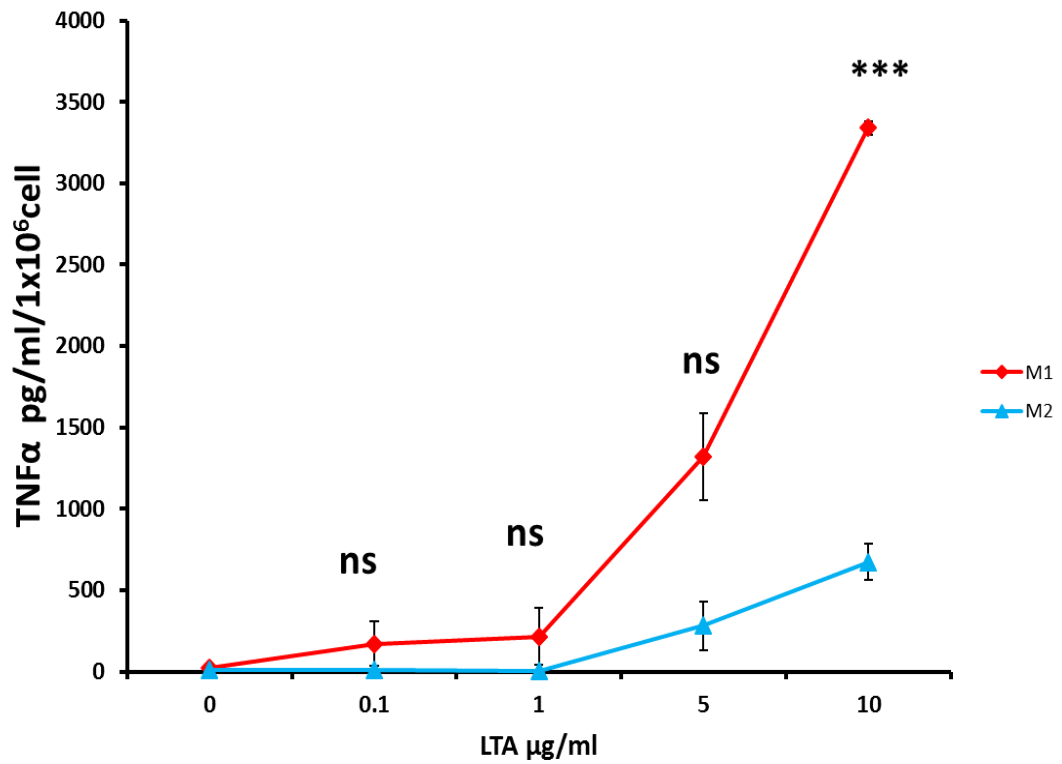
Macrophage subsets differentially respond to different concentrations of *Porphyromonas gingivalis* LPS, PG-LPS, which is associated with oral pathology. THP-1 derived macrophage subsets, M1- and M2-like, exhibited a differential response to PG-LPS by inducing comparable level of TNFα between pro-inflammatory M1 and anti-inflammatory M2 MΦs (Figure 3.2-3). TNFα production between M1- and M2-like MΦs varies depending on the concentrations used for PG-LPS stimulus. Challenge these MΦ subsets was not comparable between the two subsets when the concentration of PG-LPS is lower or higher than 0.1 µg/ml. However, stimulation of MΦ subsets by 0.1 µg/ml of PG-LPS resulted in production of comparable level of TNFα between M1 like and M2 like. TNFα production ratio between M1 like and M2 like MΦs was 2:1 and the cytokine expression was approaching significant to P= 0.05. However, ED<sub>50</sub> of PG-LPS was 0.036 µg/ml and 0.34 µg/ml for M1- and M2-like MΦs, respectively.



**Figure 3.2-3: TNF $\alpha$  production by PG-LPS stimulated M1 & M2 M $\Phi$  subsets.** THP-1-derived M1 and M2 M $\Phi$ s were generated by differentiating THP-1 monocytes with either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 3 days or 10 nM 1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> for 7 days, respectively. M1 (Red) and M2 (Blue) M $\Phi$  subsets were stimulated in the presence or absence of PG-LPS at concentration titration of [0.001, 0.01, 0.1, 0.5 and 1  $\mu\text{g/ml}$ ] for 18h. TNF $\alpha$  production is expressed as the mean  $\pm$  SD in pg/ml. Data displayed represents triplicate samples for n= 3 replicate experiments. Significant differences in cytokine production between PG-LPS activated M1 and M2 M $\Phi$ s are indicated as \*p < 0.05 and ns= not significant.

#### **3.2.1.4 Dose dependency of MΦ TNFα production upon stimulation by the TLR2 agonist, LTA.**

Macrophage subsets differentially respond to different concentrations of Lipoteichoic acid (LTA) TLR2 ligand. THP-1 derived macrophage subsets displayed a differential response to LTA by inducing comparable level of TNFα by pro-inflammatory M1 and anti-inflammatory M2 MΦs (Figure 3.2-4). TNFα production between M 1 and M 2 varies depending on the LTA stimulus concentrations used. Stimulate these MΦ subsets was not comparable between the two subsets when the concentration of LTA was lower than 10 µg/ml. However, stimulation of MΦ subsets by 10 µg/ml of LTA lead to comparable production level of TNFα between M1-like and M2-like MΦs. The relative ratio between M1-like and M2-like for ratio of 95:18 of LPS induced cytokine express over unstimulated response and  $P < 0.001$ . However,  $ED_{50}$  of LTA was 4.1 µg/ml and 6.5 µg/ml for M1- and M2-like MΦs, respectively.

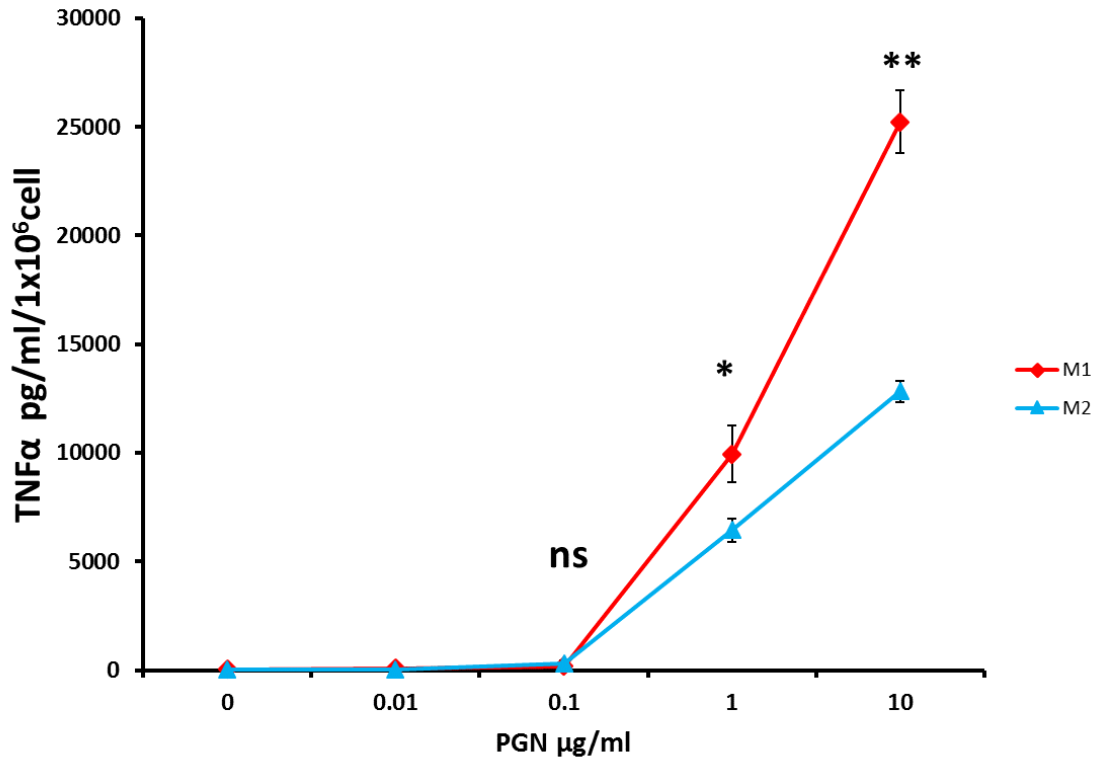


**Figure 3.2-4: TNF $\alpha$  production by LTA stimulated M1 & M2 M $\Phi$  subsets.**

THP-1-derived M1 and M2 M $\Phi$ s were generated by differentiating THP-1 monocytes with either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 3 days or 10 nM 1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> for 7 days, respectively. M1 (Red) and M2 (Blue) M $\Phi$  subsets were stimulated in the presence or absence of LTA at concentration titration of [0.01, 0.1, 1, 10  $\mu\text{g/ml}$ ] for 18h. TNF $\alpha$  production is expressed as the mean  $\pm$  SD in pg/ml. Data displayed represents triplicate samples for n= 3 replicate experiments. Significant differences in cytokine production between LTA activated M1 and M2 M $\Phi$ s are indicated as \*\*\*P < 0.001 and ns= not significant.

### **3.2.1.5      TNF $\alpha$ cytokine differentially induced by M1- & M2-like M $\Phi$ s upon NOD1/NOD2 agonist, PGN stimulation.**

Bacteria can also be sensitive PAMP upon internalisation where there is internal receptors that can recognise different forms of PGN; NOD1 and NOD2 (Chamaillard et al., 2003). Macrophage subsets differentially respond to different concentrations of peptidoglycan (PGN), NOD1/NOD2 agonist, which is associated with NF- $\kappa$ B activity. TNF $\alpha$  production by M1- and M2-like M $\Phi$ s varies depending on the concentrations used for PGN stimulus (Figure 3.2-5). Stimulation of M $\Phi$  subsets by 1  $\mu$ g/ml of PGN resulted in production of comparable level of TNF $\alpha$  between M1-like and M2-like M $\Phi$ s. The ratio of TNF $\alpha$  level by M1-like and M2 like M $\Phi$ s was 90:40 and the cytokine expression was slightly significant to  $P= 0.044$ . However, ED<sub>50</sub> of PGN was 4.75  $\mu$ g/ml and 4.6  $\mu$ g/ml for M1- and M2-like M $\Phi$ s, respectively.



**Figure 3.2-5: TNF $\alpha$  production by PGN stimulated M1 & M2 M $\Phi$  subsets.**

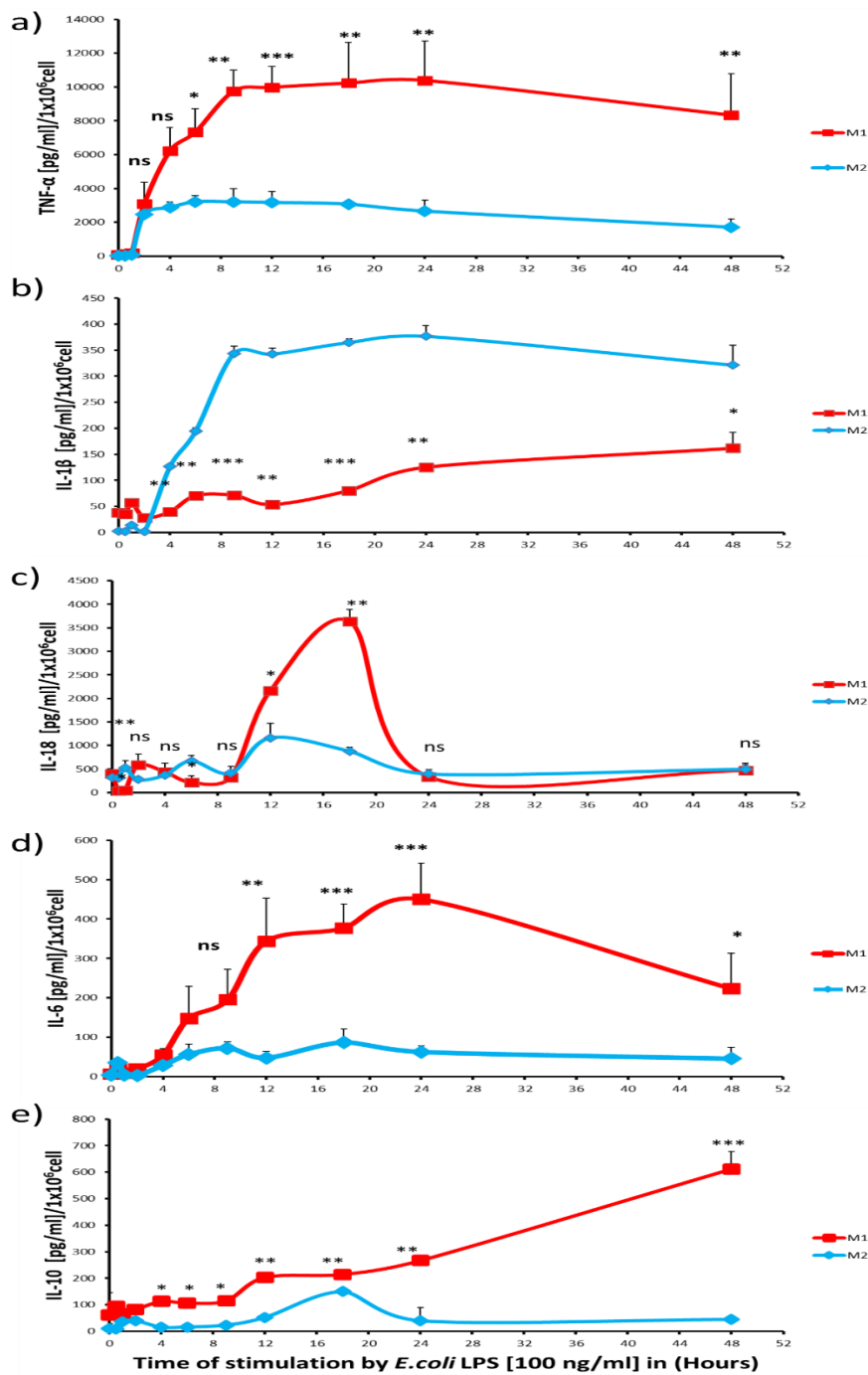
THP-1-derived M1 and M2 M $\Phi$ s were generated by differentiating THP-1 monocytes with either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 3 days or 10 nM 1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> for 7 days, respectively. M1 (Red) and M2 (Blue) M $\Phi$  subsets were stimulated in the presence or absence of PGN at concentration titration of [0.01, 0.1, 1, 10  $\mu\text{g/ml}$ ] for 18h. TNF $\alpha$  production is expressed as the mean  $\pm$  SD in pg/ml. Data displayed represents triplicate samples for n= 3 replicate experiments. Significant differences in cytokine production between PGN activated M1 and M2 M $\Phi$ s are indicated as \*p < 0.05, \*\*p < 0.01, and ns= not significant



### **3.2.1.6 Time course optimisation of cytokine production by K12-LPS stimulated MΦs subsets.**

Macrophage subsets differentially respond to *E. coli* LPS [100 ng/ml] in different time points of stimulation. Distinct cytokine profiles were exhibited by THP-1 derived macrophage subsets in response to K12-LPS. The results, as shown in (Figure 3.2-6) revealed that the cytokine production between stimulated K12-LPS M1 MΦs and M2 MΦs is time dependent. The results displayed that pro-inflammatory MΦs M1 produced higher level of TNFα, IL-18, IL-6 and IL-10 cytokines than anti-inflammatory MΦs M2. However, M2 MΦs produced higher level of IL-1β cytokine than M1 MΦs upon the time course. Over the same time point, TNFα did not go above 100pg/ml for both M1 and M2 control (data not shown). TNFα concentration induced by M1 MΦs sharply increased after 1h of incubation with stimulus from [183 pg/ml] to [9752 pg/ml] at 9h incubation. The level of TNFα production was constant after 9 h with little decrease at 48h. On the other hand, TNFα concentration produced by M2 MΦs, rose from [68 pg/ml] after 1h incubation with K12-LPS to [2468 pg/ml] at 2h incubation. Then, the level of TNFα production was continuous after 2h with slight drop at 48h to [1710 pg/ml]. The peak of TNFα cytokine productions was at 24h for M1 MΦs and at 9 h for M2 MΦs. IL-1β and IL-18 did not go above 20 and 100 pg/ml, respectively, for both M1 and M2 control over the same time point (data not shown). IL-1β concentration produced by M1 MΦs increased gradually from [28 pg/ml] at 2h incubation with K12-LPS to [163 pg/ml] at 48h. Nevertheless, IL-1β concentration induced by M2 MΦs sharply increased after 2h of incubation with K12-LPS from [2pg/ml] to [343 pg/ml] at 9h incubation. The level of IL-1β production increased

steadily to the peak at 24h before it decreased to [321 pg/ml] at 48h. The peak of IL-1 $\beta$  cytokine production was at 48h for M1 M $\Phi$ s and at 24h for M2 M $\Phi$ s. IL-18 concentration induced by M1-like and M2-like M $\Phi$ s was very low level for the first 9h. After 9h, IL-18 level increased sharply to the highest level at 3632 pg/ml by M1-like at 18h time point whereas it steadily increased to the peak of 1160 pg/ml at 12h incubation time by M2-like M $\Phi$ s. After peak time, IL-18 level dropped down in low level by both M1 and M2 M $\Phi$ s until the end of treatment time, 48h. Over the same time point, IL-6 and IL-10 did not go above 20 pg/ml for both M1 and M2 control (data not shown). IL-6 concentration induced by M1 M $\Phi$ s regularly increased after 4h of stimulation with K12-LPS from [55pg/ml] to [451 pg/ml] at 24h then it dropped to [223 pg/ml] at 48h. In contrast, IL-6 concentration produced by M2 M $\Phi$ s increased slowly to the peak of [87 pg/ml] at 18h then decreased to [62 pg/ml] and [46 pg/ml] at 24h and 48h respectively. IL-10 concentration induced by M1 M $\Phi$ s steadily increased after stimulation by K12-LPS to the peak of [611 pg/ml] at 48h incubation time. On the other hand, IL-10 production by M2 M $\Phi$ s exhibited significant different comparing to controls at only two time points; 12h and 48h with level of IL-10 equal [53 pg/ml] and [45 pg/ml], respectively. The peak of IL-10 cytokine production was at 48h for M1 M $\Phi$ s and at 12h for M2 M $\Phi$ s.

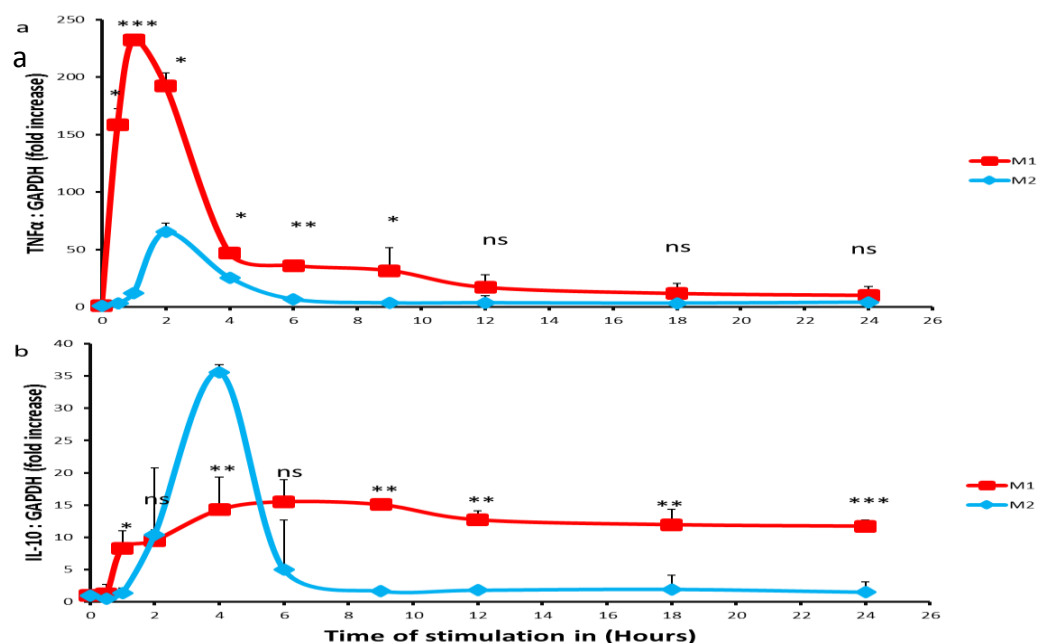


**Figure 3.2-6: Time course of cytokine production by K12-LPS stimulated M1 & M2 MΦ subsets.**

M1 (Red) and M2 (Blue) MΦ subsets were stimulated in the presence or absence of [100 ng/ml] K12-LPS in different time points [0, 30 minutes, 1, 2, 4, 6, 9, 12, 18, 24 and 48h] in density of (1x10<sup>6</sup> cells/ml). Cytokine production is expressed as the mean ± SD in pg/ml for TNFα (a), IL-1β (b), IL-18 (c), IL-6 (d) and IL-10 (e). Data displayed represents triplicate samples for n= 3 replicate experiments. Significant differences, in cytokine production between K12-LPS activated M1 and M2 MΦs in each time point, are indicated as \* p < 0.05, \*\* p < 0.01, \*\*\* P < 0.001 and ns= not significant.

### 3.2.1.6.1 Gene expression of pro-inflammatory and anti-inflammatory cytokines by K12-LPS-stimulated MΦs.

Macrophage subsets differentially respond to *E. coli* LPS in different time points of stimulation. Distinct cytokine gene expression profiles were exhibited by THP-1-derived macrophage subsets in response to K12-LPS. The results displayed that pro-inflammatory M1 MΦs produced higher level of mRNA of TNFα cytokine than anti-inflammatory M2 MΦs. However, mRNA of IL-10 in M1 MΦs seemed to be more stable in the same time points. The peak of TNFα mRNA was within 2h for M1 and M2 MΦs whereas the peak of IL-10 mRNA was within 6h and 4h for M1 MΦs and M2 MΦs, respectively (Figure 3.2-7).



**Figure 3.2-7: Gene expression of pro- and anti-inflammatory cytokines by K12-LPS-stimulated MΦs.**

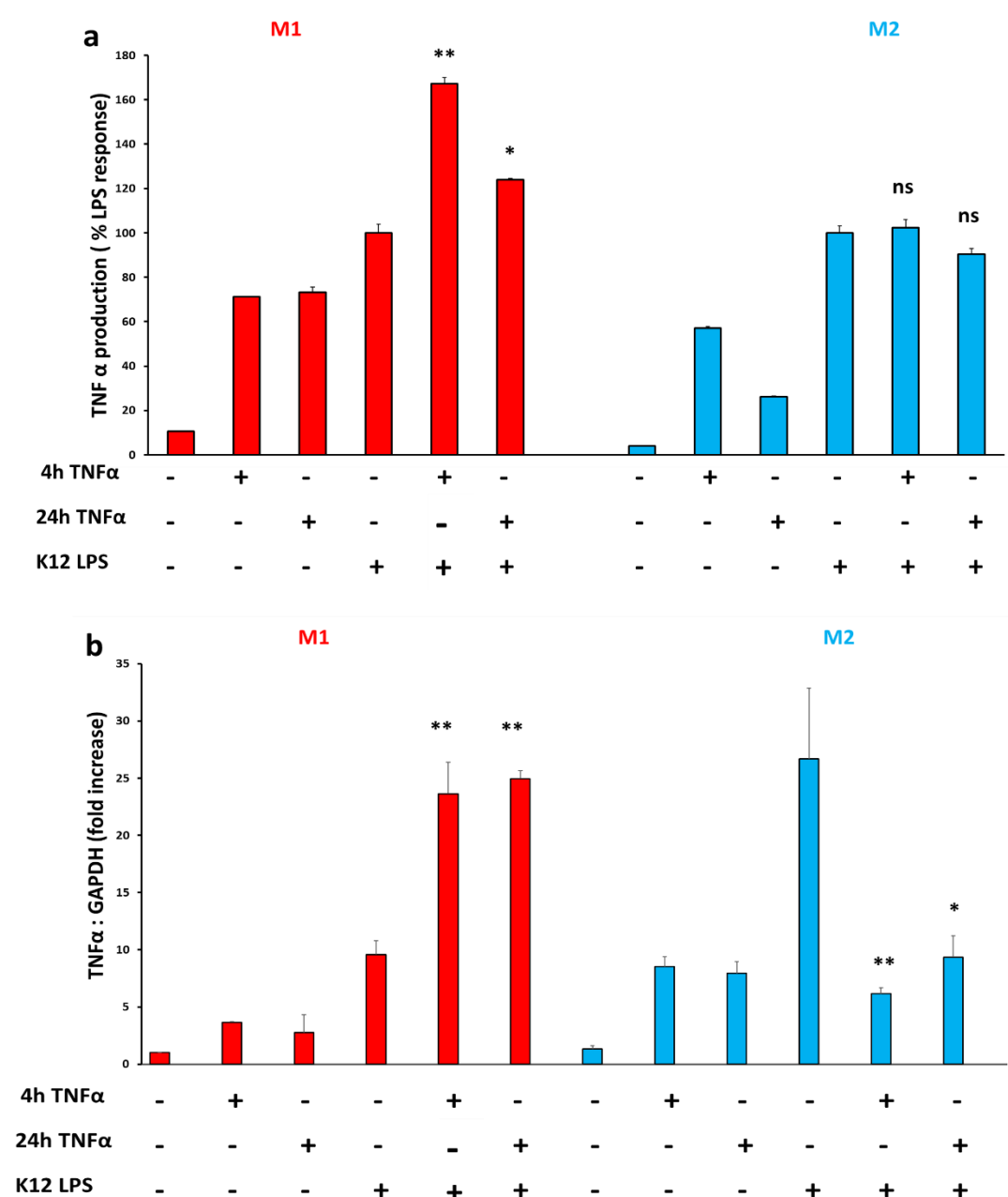
M1 (Red) and M2 (Blue) MΦ subsets were stimulated with 100 ng/ml K12-LPS for a time course (0-24h). RNA was extracted from cells to test the mRNA expression of TNFα (a) and IL-10 (b). Gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using  $2^{-\Delta\Delta Ct}$ . Data displayed is a representative experiment with duplicate samples of n=2 replicate experiments. Significant effects compared to the negative controls (unstimulated cells) are indicated as \* P< 0.05, \*\* P< 0.01 \*\*\* P< 0.001 and ns= not significant.

### **3.2.2 TNF $\alpha$ differentially modulates M $\Phi$ subject-specific responses to K12-LPS.**

In order to mimic a chronic inflammatory environment, M $\Phi$  subsets were challenged with TNF $\alpha$  for different time point prior to treatment with K12-LPS. The effect of TNF $\alpha$  on M $\Phi$  subsets were determined by quantifying cytokine production and secretion (TNF $\alpha$ , IL-18, IL-1 $\beta$ , IL-6 and IL-10). As described above, the pulse-chase method was performed to find out TNF $\alpha$  cytokine production whereas treatment without washing cells after TNF $\alpha$  stimulation was used to detect the other cytokines. Figures below showed that M $\Phi$  subsets differentially sensitive to TNF $\alpha$  stimulation in present or absent of LPS. When incubated M1-like M $\Phi$ s with TNF $\alpha$  for 4h or 24h, they are able to produce TNF $\alpha$ , although they had been washed and incubated for 18h. The level of TNF $\alpha$  released by M1-like M $\Phi$ s after 4h and 24h was 20% less than the TNF $\alpha$  produced upon LPS stimulation. Interestingly, TNF $\alpha$  was upregulated when M1-like M $\Phi$ s were challenged by TNF $\alpha$  followed by LPS and increased significantly by 70% ( $P= 0.01$ ) and 20% ( $P= 0.02$ ) upon 4h and 24h TNF $\alpha$  treatment, respectively, for a control level at 2839 pg/ml. On the other hand, the level of TNF $\alpha$  produced by M2-like M $\Phi$ s after 4h and 24h was 45% and 75% less than the TNF $\alpha$  produced upon LPS stimulation. Remarkably, there was no significant change in the level of TNF $\alpha$  produced by M2-like M $\Phi$ s after 4h and 24h compared to LPS stimulated M $\Phi$ s (Figure 3.2-8 a). The gene expression modulation of TNF $\alpha$  was reflected similarly to the modulation on secreted protein. The level of TNF $\alpha$  mRNA expressed by M1-like M $\Phi$ s after 4h and 24h was about six folds less than the TNF $\alpha$  mRNA level upon LPS stimulation. Interestingly, TNF $\alpha$  gene was upregulated when M1-like M $\Phi$ s were challenged by TNF $\alpha$ , after both pre-treatment time points, followed by LPS and increased by about 14 folds higher

than positive control. On the other hand, the level of TNF $\alpha$  produced by M2-like M $\Phi$ s after 4h and 24h was 18 fold less than the TNF $\alpha$  produced upon LPS stimulation. Remarkably, there was a significant decrease in the level of TNF $\alpha$  gene in M2-like M $\Phi$ s after 4h (by 21 fold) and 24h (18 fold) compared to LPS stimulation, (Figure 3.2-8 b). Pro-inflammatory M1-like M $\Phi$ s showed different results for IL-1 $\beta$  production in response to TNF $\alpha$  challenge. To illustrate, when incubated M1-like M $\Phi$ s with TNF $\alpha$  for 4h, IL-1 $\beta$  was released 40% less than positive control. However, there was no significant change in the level of IL-1 $\beta$  production when M1-like M $\Phi$ s were treated with TNF $\alpha$  only for 24h nor when pre-treated with TNF $\alpha$  for 4h and 24h followed by 18h LPS stimulation. The control level was 611 pg/ml. Anti-inflammatory M2-like M $\Phi$ s did not release IL-1 $\beta$  in response to 4h TNF $\alpha$  challenge but it produced 40% IL-1 $\beta$  less than IL-1 $\beta$  produced upon LPS stimulation. IL-1 $\beta$  was down-regulated when M2-like M $\Phi$ s were challenged by TNF $\alpha$  followed by LPS and decreased significantly by 40% (P= 0.003) and 70% (P= 0.002) upon 4h and 24h TNF $\alpha$  treatment, respectively, for a control level at 668 pg/ml (Figure 3.2-9 a). The level of IL-18 produced by M1-like, after 4h and 24h of TNF $\alpha$  challenge, was roughly 30% less than IL-18 produced upon LPS stimulation. However, LPS-stimulated M1-like M $\Phi$ s produced higher amount of IL-18 upon pre-treatment with TNF $\alpha$ . The upregulation obtained after 4h and 24h of pre-treatment was significant as 25% (P=0.002) and 45% (P= 0.001), respectively, compared to a control level at 3794 pg/ml. The level of IL-18 produced by M2-like, after 4h and 24h of TNF $\alpha$  challenge, was 90% less than the IL-18 level produced upon LPS stimulation. Interestingly, pre-stimulation with TNF $\alpha$  contribute to suppress the production of IL-18 by LPS-stimulated M2-like M $\Phi$ s. The observed suppression was 35% (P= 0.001) after 4h and 45% (P= 0.006) after 24h of TNF $\alpha$  pre-treatment (Figure 3.2-9 b) where the control level was 1842 pg/ml. M1-like M $\Phi$ s produced very low level of IL-6 production

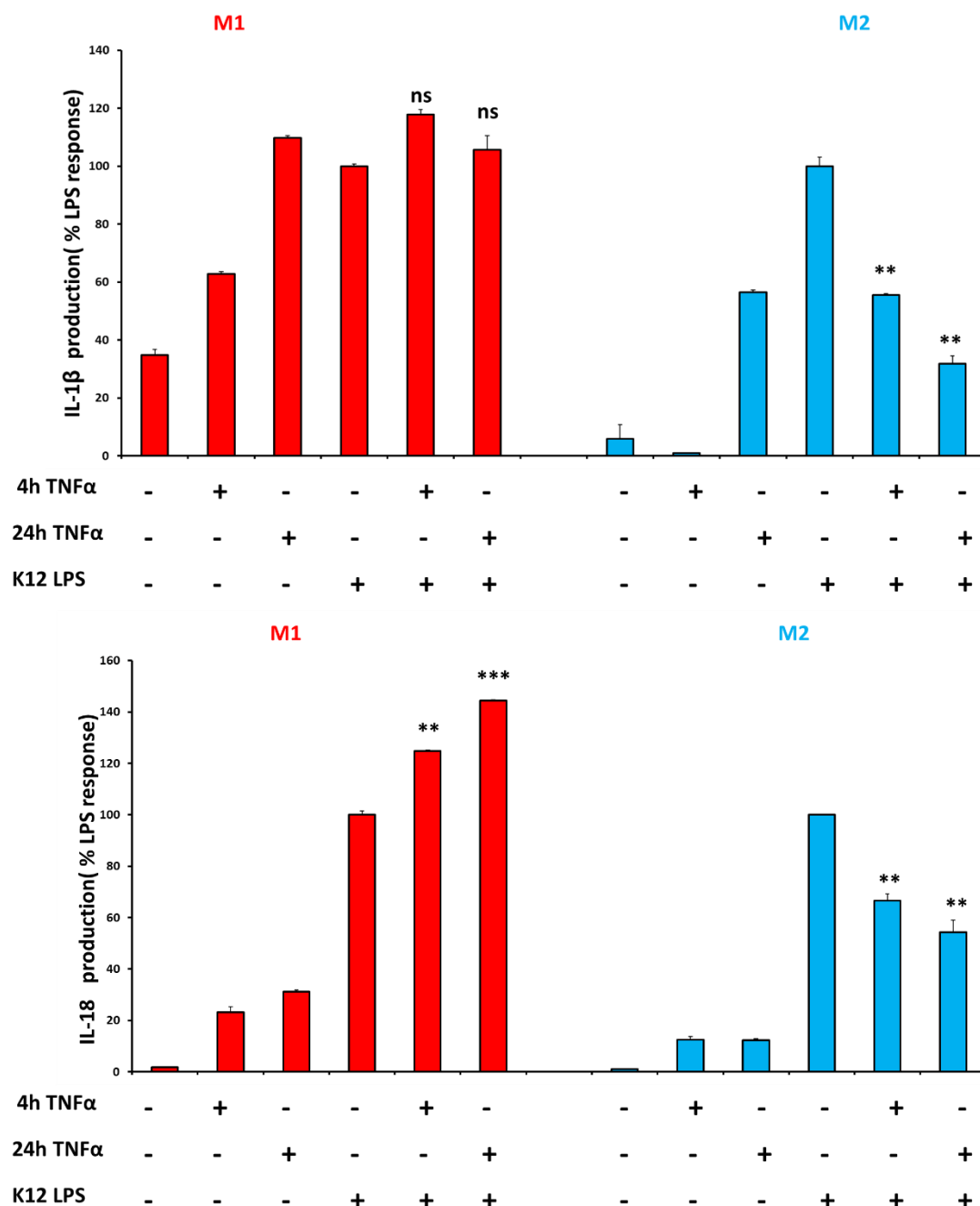
upon 4h and 24h TNF $\alpha$  treatment, which was similar to unstimulated cells. Interestingly, pre-treatment with TNF $\alpha$  followed by LPS stimulation enhanced M1-like M $\Phi$ s to increase the production of IL-6. The upregulation was 72% ( $P= 0.14$ ) and 120% ( $P< 0.001$ ) higher than positive control for 4h and 24h pre-treatment, respectively. The level of IL-6 produced by M2-like M $\Phi$ s after TNF $\alpha$  treatment for 4h and 24h was 40% and 60% less than the IL-6 produced upon LPS stimulation. There was no significant change in the level of IL-6 production when M2-like M $\Phi$ s were pre-treated with TNF $\alpha$  for 4h prior to LPS stimulation ( $P= 0.433$ ), whereas treatment for 24h before LPS stimulation enhanced IL-6 production by 140% ( $P= 0.014$ )(Figure 3.2-10 a). The control level was 27 pg/ml for M1-like and 50 pg/ml for M2-like M $\Phi$ s. Anti-inflammatory cytokine, IL-10, was only released after 24h of TNF $\alpha$  treatment by M1-like M $\Phi$ s by 20% less than IL-10 produced upon LPS stimulation. Interestingly, LPS-stimulated M1-like M $\Phi$ s significantly decreased IL-10 production by 30% upon TNF $\alpha$  pre-treatment for 4h ( $P= 0.042$ ) and 24h ( $P= 0.016$ ) for a control level at 133 pg/ml. IL-10 was released only after 24h of TNF $\alpha$  treatment by M2-like M $\Phi$ s by 30% less than IL-10 produced upon LPS stimulation. IL-10 cytokine was upregulated by 25% ( $P= 0.433$ ) and 70% ( $P= 0.014$ ) upon 4h and 24h TNF $\alpha$  treatment, respectively, when M2-like M $\Phi$ s were challenged by TNF $\alpha$  prior to LPS as the control level was 41 pg/ml (Figure 3.2-10 b).



**Figure 3.2-8: Differential sensitivity of K12-LPS-induced MΦ TNFα to TNFα.**

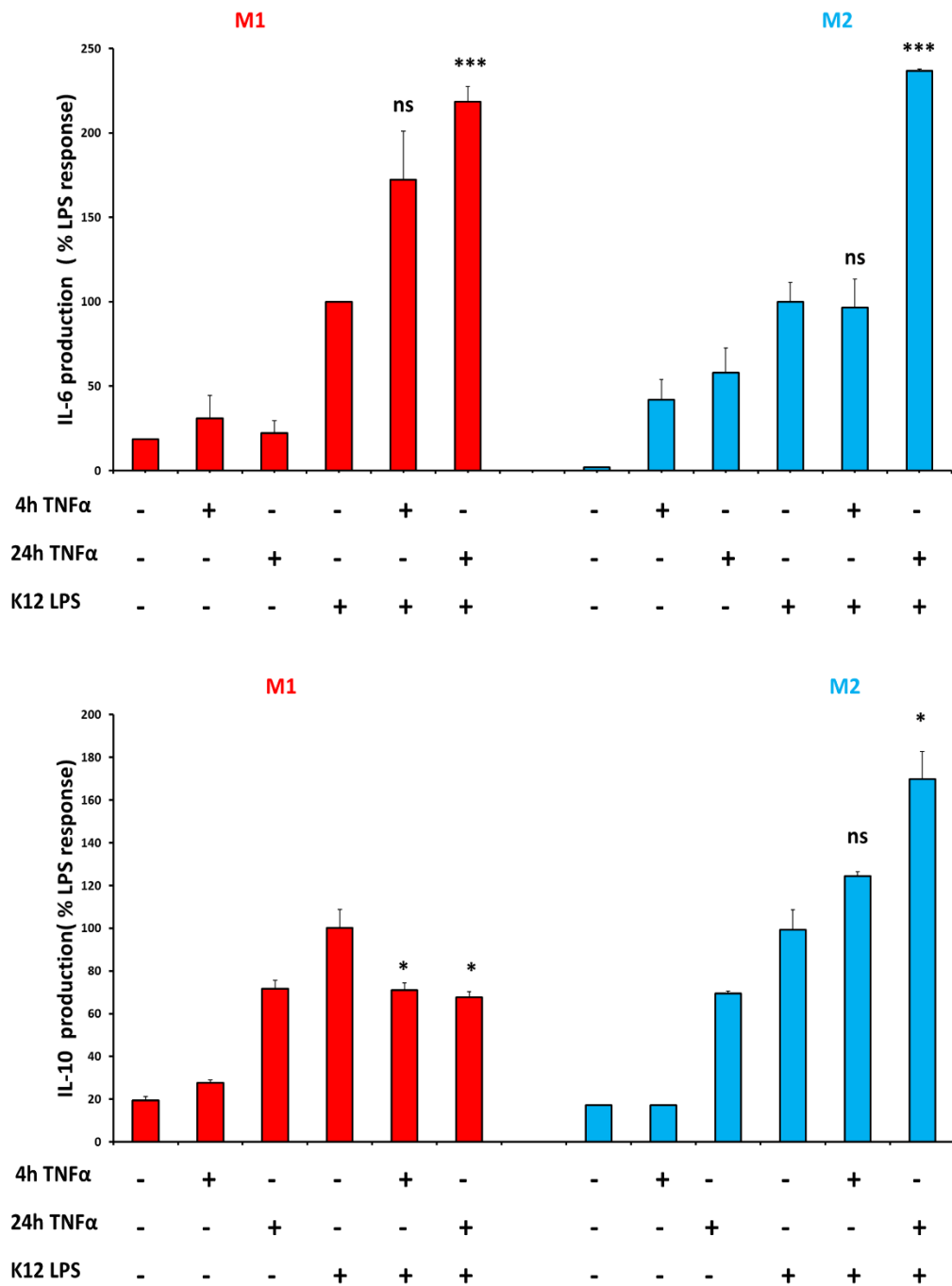
M1 (Red) and M2 (Blue) MΦ subsets were stimulated by 10 ng/ml TNFα for 4h and/or 24h followed by washing the cells with fresh medium and incubating with or without 100 ng/ml LPS for 18h. TNFα production (a) is expressed as the mean ± SD in pg/ml. RNA was extracted from cells to test the mRNA expression of TNFα (b). Gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using  $2^{-\Delta\Delta Ct}$ . Data displayed represents duplicate samples for n= 2 replicate experiments. Significant differences in cytokine production between LPS activated M1 and M2 MΦs and TNFα/LPS treated MΦs are indicated as \*p< 0.05, \*\*p< 0.01 and ns= no significant.





**Figure 3.2-9: Differential sensitivity of K12-LPS-induced MΦ IL-1β and IL-18 to TNFα.**

M1 (Red) and M2 (Blue) MΦ subsets were stimulated by 10 ng/ml TNFα for 4h and/or 24h followed by incubating with or without 100 ng/ml LPS for 18h. IL-1β (a) and IL-18 (b) production is expressed as the mean  $\pm$  SD in pg/ml. Data displayed represents duplicate samples for n= 2 replicate experiments. Significant differences in cytokine production between LPS activated M1 and M2 MΦs and TNFα/LPS treated are indicated as \*\*p < 0.01, \*\*\*P < 0.001 and ns= not significant.



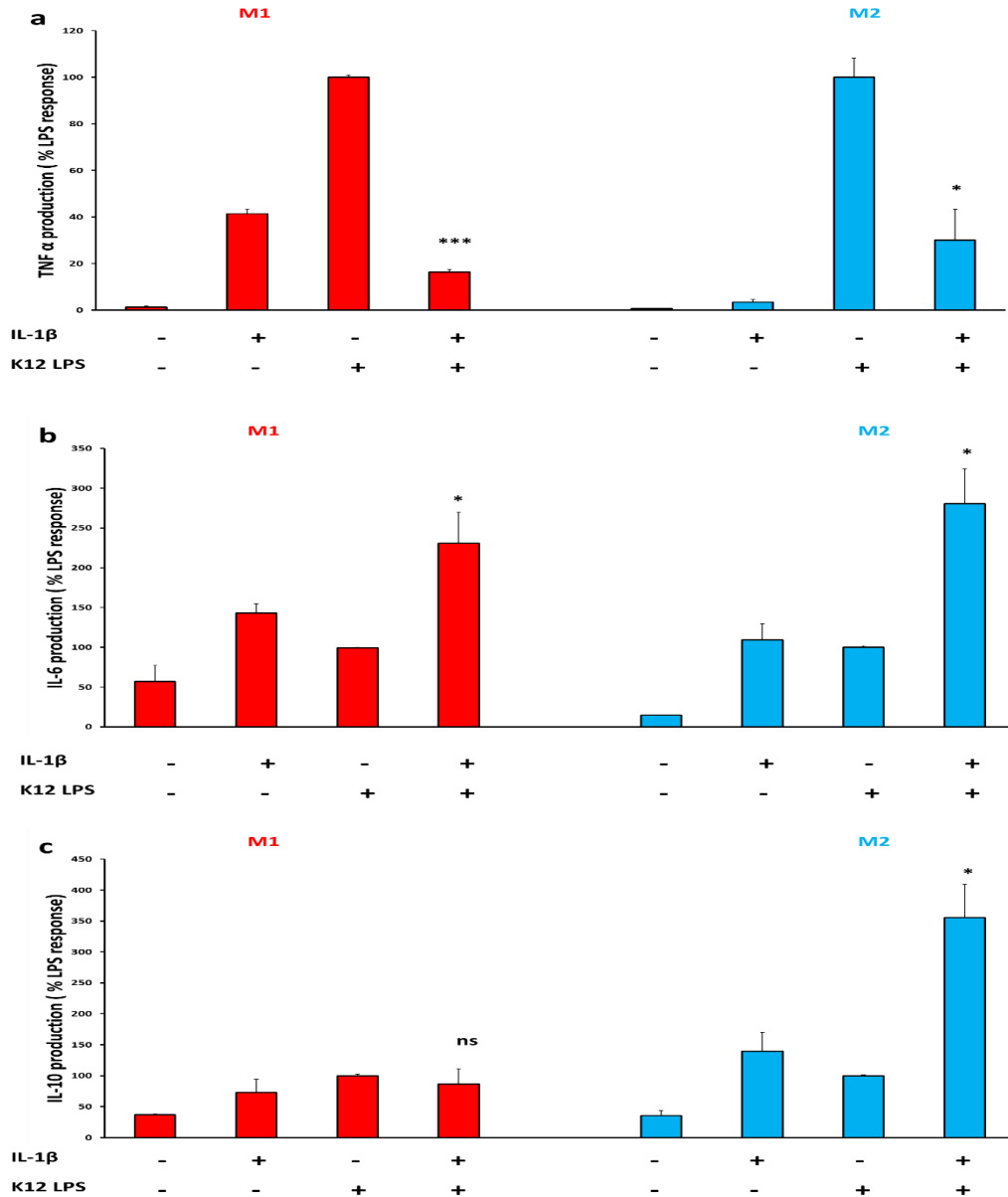
**Figure 3.2-10: Differential sensitivity of K12-LPS-induced MΦ IL-6 and IL-10 to TNFα.**

M1 (Red) and M2 (Blue) MΦ subsets were stimulated by 10 ng/ml TNFα for 4h and/or 24h followed by incubating with or without 100 ng/ml LPS for 18h. IL-6 (a) and IL-10 (b) production is expressed as the mean  $\pm$  SD in pg/ml. Data displayed represents duplicate samples for  $n = 2$  replicate experiments. Significant differences in cytokine production between LPS activated M1 and M2 MΦs and TNFα/LPS treated are indicated as \* $p < 0.05$ , \*\*\* $p < 0.001$  and ns= not significant.

### 3.2.3 Differential sensitivity of K12-LPS-induced MΦ cytokines to IL-1β.

In order to mimic a chronic inflammatory environment, MΦ subsets were challenged with IL-1β for a specific time (24h) before treatment with K12-LPS. The effect of IL-1β on MΦ subsets was determined by quantifying cytokine production (TNFα, IL-6 and IL-10). Figures below showed that MΦ subsets were differentially modulated by IL-1β upon stimulation by LPS. It was shown above that M1 and M2 MΦs in response to LPS stimulation can secrete pro- and anti-inflammatory cytokines such as TNFα and IL-10. However, when MΦ subsets were stimulated with IL-1β the produced cytokine profile was different (Figure 3.2-11). Overall, M1-like MΦs secreted 934 pg/ml TNFα in response to IL-1β challenge whereas M2-like MΦs did not show any significant production of TNFα 31 pg/ml only compared to TNFα production upon LPS stimulation. Compared to a control of LPS stimulation at 2255 pg/ml, TNFα production by M1-like MΦs decreased significantly by 84% (P= 0.001) upon 24h of IL-1β stimulation prior to LPS stimulation. Additionally, TNFα production by M2-like MΦs decreased slightly significant by 70% (P= 0.02) compared to a control of LPS stimulation at 918 pg/ml (Figure 3.2-11 a). Interestingly, both M1- and M2-like produced cytokines in response to IL-1β. Upon IL-1β stimulation, M1-like and M2-like MΦs produced higher level of IL-6 by 115 pg/ml and 59 pg/ml, respectively. The control level was 80 pg/ml for M1-like and 54 pg/ml for M2-like MΦs. Remarkably, IL-6 production significantly increased by 131% (P=0.04) by M1-like MΦs and 181% (P= 0.03) M2-like MΦs upon 24h of IL-1β stimulation prior to LPS stimulation (Figure 3.2-11 b). Dissimilar readout for IL-10 cytokine by both MΦ subsets. IL-1β stimulated-M1 MΦs produced lower IL-10 (135 pg/ml) and LPS-stimulated M1 produced (185 pg/ml). However, M2 MΦs produced higher IL-10 (210 pg/ml) upon IL-1β challenge and (150 pg/ml) upon LPS stimulation. M1-like MΦs produced IL-10 cytokine upon 24h of IL-1β stimulation prior to LPS

stimulation at similar level of LPS stimulation. However, IL-10 cytokine 265% significantly higher by M2-like MΦs upon 24h of IL-1β stimulation prior to LPS stimulation ( $P = 0.02$ ) for the level of control at 150 pg/ml (Figure 3.2-11 c).

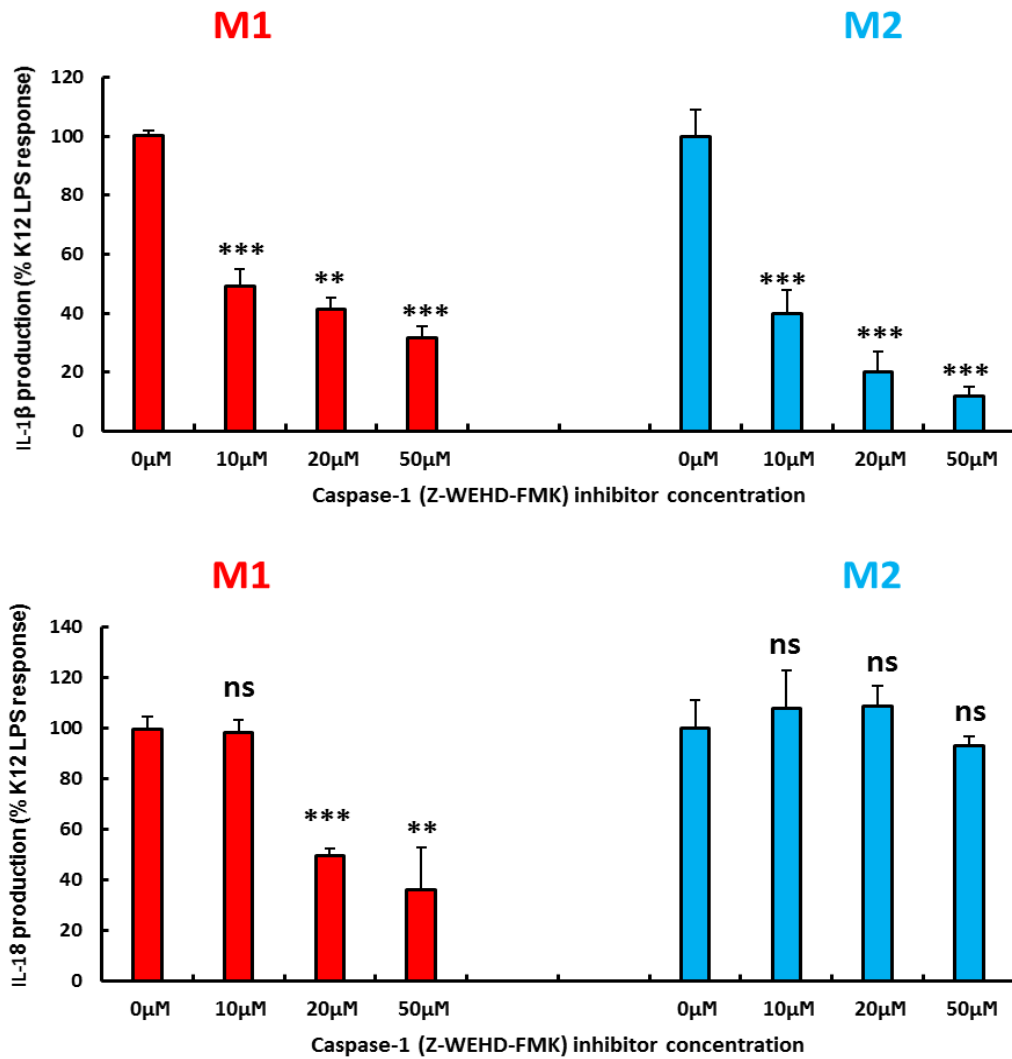


**Figure 3.2-11: Differential sensitivity of K12-LPS-induced MΦs cytokines to IL-1β.**

M1 (Red) and M2 (Blue) MΦ subsets were pre-treated with 10 ng/ml IL-1β for 24h followed by 18h incubation with or without 100 ng/ml LPS. TNFα, IL-6 and IL-10 production is expressed as the mean  $\pm$  SD in pg/ml. Data displayed represents duplicate samples for  $n = 2$  replicate experiments. Significant differences in cytokine production between LPS activated M1 and M2 MΦs and IL-1β /LPS treated are indicated as \* $p < 0.05$ , \*\*\* $P < 0.001$  and ns= not significant.

### **3.2.4 M1 and M2 MΦs secretion of IL-1 $\beta$ and IL-18 display a differential dependence on caspase-1.**

As shown previously, MΦ subsets are able to display inflammasome activation upon K12-LPS stimulation. To investigate whether IL-1 $\beta$  and IL-18 secretion is caspase-1 dependent from LPS-activated MΦs, IL-1 $\beta$  and IL-18 secretion was examined by LPS-activated MΦs with and without caspase-1 (Z-WEHD-FMK) inhibitor. Range of concentration of caspase-1 inhibitor was used in this study between 10  $\mu$ M and 50  $\mu$ M. The production of IL-1 $\beta$  significantly dropped upon treatment with 10  $\mu$ M of caspase-1 inhibitor before LPS stimulation by both MΦ subsets, M1 and M2, by 50% ( $P= 0.002$ ) and 60% ( $P= 0.001$ ), respectively. IL-1 $\beta$  secretion by both MΦ subsets was decreased gradually in parallel with the increase of caspase-1 inhibitor concentration. The level of IL-1 $\beta$  after treating with 50  $\mu$ M before LPS stimulation was significantly low by 68% less ( $P< 0.001$ ) for M1-like and 88% less ( $P= 0.003$ ) for M2-like MΦs. The control level was 273 pg/ml for M1-like and 343 pg/ml for M2-like MΦs. Moreover, 50% inhibitory control test (ICT) of caspase-1 (Z-WEHD-FMK) inhibitor was 48  $\mu$ M for M1-like MΦs and 32.5  $\mu$ M for M2-like MΦs. Interestingly, the case with IL-18 production was different i.e. IL-18 cytokine seems to be caspase-1 dependent by M1-like MΦs whereas it is not associated with caspase-1 in M2-like MΦs. To illustrate, M1-like MΦs reduced IL-18 production by 50%, when 20 and 50  $\mu$ M caspase-1 inhibitor were used before LPS stimulation as ( $P< 0.001$ ) for 50  $\mu$ M treatment and ( $P= 0.003$ ) for 20  $\mu$ M treatment, where the control was 152 pg/ml. On the other hand, M2-like MΦs showed no significant change in the level of IL-18 production using a caspase-1 inhibitor before LPS Stimulation.



**Figure 3.2-12: Caspase 1 inhibitor differentially modulates IL-1 $\beta$  and IL-18 secretion by LPS-activated M $\Phi$ s.**

M1-like M $\Phi$  (red) and M2-like M $\Phi$  (blue) were incubated with range of concentration (0, 10, 20 and 50  $\mu$ M) of caspase-1 (Z-WEHD-FMK) inhibitor for 1h prior to stimulation for 18h with or without 100 ng/ml LPS. IL-1 $\beta$  and IL-18 production is expressed as the mean  $\pm$  SD% LPS induced for cytokine production. Data displayed represents duplicate samples for n= 2 replicate experiments. Significant differences in cytokine production between activated M1- and M2-like M $\Phi$ s are indicated as \*\*p < 0.01, \*\*\*p < 0.001 and ns= not significant.

### 3.3 Summary of chapter 3 results

**Table 3.3-1: Cytokine production by 100 ng/ml K12-LPS stimulated M1 & M2 MΦ subsets.**

	M1-like MΦ		M2-like MΦ	
	Cytokine Level Pg/ml	ED <sub>50</sub>	Cytokine Level Pg/ml	ED <sub>50</sub>
<i>TNFα</i>	3096	0.482 µg/ml	806	0.45 µg/ml
<i>IL-1β</i>	126	0.25 µg/ml	230	0.25 µg/ml
<i>IL-18</i>	8512	0.030 µg/ml	2031	0.035 µg/ml
<i>IL-6</i>	254	0.41 µg/ml	90	0.41 µg/ml
<i>IL-10</i>	327	0.17 µg/ml	13	0.12 µg/ml

Table shows the cytokine production [pg/ml] upon stimulation with 100 ng/ml K12-LPS and ED<sub>50</sub> obtained from concentration titration of K12-LPS [0.001, 0.01, 0.1, 0.5 and 1 µg/ml].

**Table 3.3-2: TNFα production by M1 & M2 MΦ subsets stimulated with different PAMPs.**

	M1-like MΦs		M2-like MΦs	
	Cytokine Level	ED <sub>50</sub>	Cytokine Level	ED <sub>50</sub>
<b><i>K12-LPS [100 ng/ml]</i></b> <b><i>(TLR4)</i></b>	3096	0.482 µg/ml	806	0.45 µg/ml
<b><i>PG-LPS [100 ng/ml]</i></b> <b><i>(TLR2/4)</i></b>	1694	0.036 µg/ml	799	0.34 µg/ml
<b><i>PGN [10 µg/ml]</i></b> <b><i>(NOD2)</i></b>	25293	4.75 µg/ml	12820	4.6 µg/ml
<b><i>LTA [10 µg/ml]</i></b> <b><i>(TLR2)</i></b>	3340	4.1 µg/ml	674	6.5 µg/ml

Table shows TNFα cytokine production [pg/ml] upon stimulation with PAMPs and ED<sub>50</sub> obtained from concentration titration of indicated PAMPs.

**Table 3.3-3: Differential sensitivity of K12-LPS-induced MΦs cytokines to TNFα**

	4h TNFα before (LPS): LPS only (%)	
<i>TNFα</i>	67% ↑	2% ↔
<i>TNFα mRNA</i>	130% ↑	24% ↓
<i>IL-18</i>	25% ↑	33% ↓
<i>IL-1 β</i>	18% ↑	44% ↓
<i>IL-6</i>	72% ↑	3% ↔
<i>IL-10</i>	29% ↓	24% ↑
	24h TNFα before (LPS): LPS only (%)	
<i>TNFα</i>	24% ↑	10% ↔
<i>TNFα mRNA</i>	140% ↑	39% ↓
<i>IL-18</i>	44% ↑	46% ↓
<i>IL-1 β</i>	6% ↔	68% ↓
<i>IL-6</i>	119% ↑	137% ↑
<i>IL-10</i>	32% ↓	70% ↑

Percentage (%) change in LPS response. Note: “↑”, “↓,” and “↔” means up-regulation, down-regulation, and no-modulation of the indicated target, respectively.



**Table 3.3-4: Differential sensitivity of K12-LPS-induced MΦs cytokines to IL-1β.**

	M1-like MΦ	M2-like MΦ
	24h IL-1β before LPS (%)	
<b><i>TNFα</i></b>	41% ↓	97% ↓
<b><i>IL-6</i></b>	45% ↑	8.5% ↔
<b><i>IL-10</i></b>	27% ↓	29% ↑

*Note: “↑”, “↓,”and “↔”means up-regulation, down-regulation, and no-modulation of the indicated target, respectively.*

**Table 3.3-5: The effect of Caspase 1 inhibition on K12-LPS-induced MΦs**

	M1-like MΦ		M2-like MΦ	
	IL-1β	IL-18	IL-1β	IL-18
<b><i>LPS only</i></b>	100%	100%	100%	100%
<b><i>10 μM* + LPS</i></b>	51% ↓	2% ↓	60% ↓	8% ↑
<b><i>20 μM* + LPS</i></b>	59% ↓	50% ↓	80% ↓	9% ↑
<b><i>50 μM* + LPS</i></b>	68% ↓	64% ↓	88% ↓	7% ↓

*(\*) Caspase-1 inhibitor concentrations added before LPS inhibitor + LPS*

*Note: “↑”, “↓,”and “↔”means up-regulation, down-regulation, and no-modulation*

## 3.4 Discussion

### 3.4.1 THP-1 monocytes differentiated macrophage subsets and cytokines profiles

There are several conclusions from this investigation with respect to MΦ subsets behaviour in responses to PAMPs include K12-LPS, PG-LPS, PGN and LTA and pro-inflammatory cytokines such as TNFα and IL-1β. Numerous studies have verified that gut macrophages have two subsets, classically activated pro-inflammatory M1 MΦ and alternative anti-inflammatory M2 MΦ (Gordon, 2003, Mosser and Edwards, 2008). Using THP-1 human monocytic cell line to generate macrophage cell subsets as a model in this study demonstrated the validation of different distinct macrophage cell phenotypes representative of M1-like, and M2-like macrophages. The cytokines TNFα, IL-1β and IL-6 that are a main cytokines of gut pathology (Zuo et al., 2010) as well as IL-18 and IL-10, were used as indicators to discriminate between macrophage subsets in response to K12-LPS stimulation. Indeed, cytokine readout showed that monocytes differentiated by PMA, M1-like, varied from monocytes differentiated by Vitamin D<sub>3</sub>, M2-like, producing different cytokine levels, and profiles. Firstly, the PAMP-induced profile of pro-inflammatory and anti-inflammatory cytokine production is dependent on the route of MΦ differentiation. Overall, in response to K12-LPS, M1-like MΦs were characterised as TNFα<sup>hi</sup>, IL-1β<sup>lo</sup>, IL-18<sup>hi</sup> and IL-6<sup>hi</sup>, whereas M2-like MΦs were TNFα<sup>lo</sup>, IL-1β<sup>hi</sup> and IL-18 and IL-6<sup>lo</sup>. The induction of the anti-inflammatory cytokine, IL-10, by M1-like MΦs was higher than M2-like MΦs. Secondly, M1-like MΦs were characterised as TNFα<sup>hi</sup> and M2-like MΦs were TNFα<sup>lo</sup> when challenge with either PG-

LPS, PGN or LTA. To produce similar amount of TNF $\alpha$  upon stimulation by different PAMPs, the concentration of these PAMPs was various.

In this study, K12-LPS was able to activate inflammasome in M $\Phi$  subsets and the readout was the production of pro-inflammatory cytokines IL-1 $\beta$  and IL18. PMA alone was able to encourage IL-1 $\beta$  secretion by M1. It was suggested that pro-IL-1 $\beta$  induction is provoked either by a short stimulation with LPS or by stimulation of THP-1 maturation into macrophage-like cells with PMA (Tsuchiya et al., 1982). Briefly, IL-1 $\beta$  augmentation and inflammatory caspase initiation was specially persuaded upon mechanical disruption of the integrity of THP-1 cells and noticed in the subsequent cytoplasmic fractions. As they resemble primary monocytes, THP-1 cells were applied where they constitutively express high levels of caspase-1, caspase-5, NALP1 and 3 (Martinon et al., 2002). This finding can explain the reduction of IL-1 $\beta$  production upon using caspase-1 inhibitor as discussed below.

This finding supports that LPS is capable to activate inflammasome and encourages both M $\Phi$  subsets to release IL-1 $\beta$  and IL18 (Dinarello et al., 2013a). In contrast to our understanding of pro-inflammatory and anti-inflammatory M $\Phi$  subsets, M2-like M $\Phi$ s produce higher levels of IL-1 $\beta$  in response to *E. coli* LPS. It is suggested that IL-1 $\beta$  is involved in mediating anti-inflammatory responses via its ability to induce IL-10 expression (Foey et al., 1998). As described below, it is interesting to note that M1-like M $\Phi$ s produced a considerable amount of the pro-inflammatory cytokine, TNF $\alpha$ , in response to IL-1 $\beta$  challenges, whereas M2-like M $\Phi$ s did not.

Although it is believed that IL-6 is a pro-inflammatory cytokine, it has been described that IL-6 showed anti-inflammatory properties. The nature of IL-6 production and function is probably reflected in different signalling profiles (Tilg et al., 1994). Anti-

inflammatory effects of IL-6 is reflected over initiation of SOCS proteins and STAT-3 activation (Xing et al., 1998) and reviewed in (Heinrich et al., 2003). In fact, SOCS-3 is associated with M1 classical MΦ polarisation and can suppress the anti-inflammatory signal and expression of IL-6 and IL-10. On the other hand, SOCS-3 knockdown favours M2 polarisation (Liu et al., 2008). Therefore, the mutual association between SOCS-3 and STAT-3 would seem to balance pro- or anti-inflammatory outcome of IL-6 and the polarisation of MΦs between M1 and M2 subsets. M1 MΦs produced significantly high level of IL10, although it is expected that the higher level of IL-10 production will be by M2 MΦs. The high release of IL-10 from M1 MΦs might be elucidated by the point that LPS is known to induce IL-10 production in human macrophages differentiated with M-CSF (Kwan et al., 2007). However, it has been observed in a previous study that there is a chance that IL-10 in M2 MΦs is expressed endogenously or as membrane bound protein (Fleming and Campbell, 1996). Additionally, IL-10 mRNA is less stable in M2s than M1s as shown in gene expression results (Figure 3.2-7 b). As M1 MΦs display  $\text{TNF}\alpha^{\text{hi}}$ ,  $\text{IL-1}\beta^{\text{lo}}$ ,  $\text{IL-6}^{\text{lo}}$ ,  $\text{IL-10}^{\text{lo}}$  and  $\text{NF-}\kappa\text{B}^{\text{hi}}$ , they are demonstrative of recruited and pro-inflammatory pathological MΦs. However, M2 MΦs, which demonstrate  $\text{TNF}\alpha^{\text{lo}}$ ,  $\text{IL-1}\beta^{\text{hi}}$ ,  $\text{IL-6}^{\text{hi}}$ ,  $\text{IL-10}^{\text{hi}}$ ,  $\text{TGF}\beta^{\text{hi}}$  and  $\text{NF-}\kappa\text{B}^{\text{lo}}$ , they are demonstrative of regulatory, anti-inflammatory mucosal MΦs. Moreover, mucosal MΦs are described to occur in these distinct functional subsets, administrated by environmental stimuli (Foey, 2012, Smythies et al., 2005).

### **3.4.2 $\text{TNF}\alpha$ differentially modulate LPS-induced MΦ subset cytokine.**

As well known that macrophages exhibit a variety of functional features, which include establishment of inflammation, tissue repair and anti-inflammatory responses or immune-suppression (Foey, 2014). Mucosal MΦs drive tolerogenic behaviour in a

homeostatic environment while, at the same time, maintaining an effective phagocytic reaction. As mentioned before, pro-inflammatory M1 MΦs encourage inflammation and have a damaging effect on tissues, whereas anti-inflammatory/regulatory M2 MΦs induce convenient effects on the tissues and reduce inflammation (Weber et al., 2015). It is suggested that TNFα has a vital role in modulation of inflammation of IBD whereas TNFα inhibition will play an essential role in Crohn's disease. While LPS activation can lead to pro- and anti-inflammatory cytokine production, these responses can be modulated by other factors. From that knowledge, it was hypothesised that THP-1 monocytes differentiated macrophage subsets, M1-like and M2-like may differentially respond to TNFα challenge long term or short term with or without LPS. TNFα-induced innate activation in the presence or absence of LPS to investigate the segregation of a pro- and an anti-inflammatory cytokine in MΦ subsets. Certainly, pro-inflammatory cytokines, TNFα, as expected, differentially modulated K12-LPS induced cytokine production in macrophage subsets. In the case of an inflammatory environment (TNFα long-term stimulation), M1-like MΦs were able to produce more pro-inflammatory cytokines such as TNFα and IL-18 and less anti-inflammatory cytokines, IL-10, compared to LPS control. M2-like MΦs, on the other hand, produced less pro-inflammatory cytokines such as TNFα and IL-18 and high anti-inflammatory cytokines, IL-10. Thus, the specific TNFα activation through its receptors was shown to increase inflammation via M1-like by production of pro-inflammatory cytokines, in the presence of LPS to mimic inflammatory environment. In contrast, M2-like MΦs showed remarkable tolerance induction. It is suggested that TNF α signal transduction pathway may converge, through activation of TRAF2, at the level of NF-κB inducing kinase leading to nuclear translocation of NF-κB. It could be that down-regulation of this pathway is a primary mechanism for interaction between TNFα and

K12-LPS, since potential post-receptor convergent signal transduction pathways are triggered in reaction to LPS and TNF $\alpha$  (Song et al., 1997). Ferlito et al verified that pre-treatment with TNF $\alpha$  in THP-1 cells line can induces a degree of cross-tolerance to LPS. The TNF $\alpha$  tolerance suppressed LPS-induced ERK phosphorylation and encouraged an NF- $\kappa$ B-binding pattern involving of augmented p50 homodimers similar to LPS tolerance in addition to decreased LPS-induced TxB2 production (Ferlito et al., 2001). Other studies have proven that pre-treatment with TNF $\alpha$  *in vivo* leaded to cross-tolerance to LPS-induced lethality in rats and encouraging macrophage functional phenotype typical of LPS tolerance (Zingarelli et al., 1995). Interestingly, the upregulation of IL-6 secretion by both macrophage subsets might explain the dual function of IL-6 cytokine as pro- and anti-inflammatory cytokine.

### **3.4.3 IL-1 $\beta$ differentially modulate LPS-induced M $\Phi$ subset cytokine.**

As mentioned above, it was remarkable that M $\Phi$  subsets deferentially response to IL-1 $\beta$  stimulation. To illustrate, M1s produced a higher amount of TNF $\alpha$  in response to IL-1 $\beta$  stimulation whereas M2s did not produce any considerable TNF $\alpha$ . It cannot be assumed that IL-1 $\beta$  cytokine could not stimulate M2s because M2-like M $\Phi$ s produced significant level of IL-6 similar to level upon LPS stimulation and even higher level of IL-10 than positive control. It might be that IL-1 $\beta$  cytokine is not associated with downstream signalling events that lead to activate TNF $\alpha$  synthesis. This demonstration was also found in study by Ferlito et al who aproved that IL-1 $\beta$  does not induce TNF  $\alpha$  in THP-1 cells (undifferentiated). It was also reported that IL-1 $\beta$  did not display an activation in NF- $\kappa$ B-DNA and did not result in degradation of I $\kappa$ B $\alpha$  which was degraded by LPS stimulation (Ferlito et al., 2001). This may be related similarly to Vit.D<sub>3</sub> differentiated THP-1, M2-like M $\Phi$ s that was used in this study.

#### **3.4.4 Inflammasome activation in response to K12-LPS stimulation.**

Finally, inflammasome activity in MΦ subsets has been investigated by several methods such as quantifying the production of IL-1β and IL-18 in response to K12-LPS, determination of sensitivity of K12-LPS-induced MΦs cytokines to IL-1β and investigating the effect of caspase-1 inhibitor on IL-1β and IL-18 production by LPS-stimulated MΦ subsets. It was demonstrated that IL-1β and IL-18 produced in different processes in LPS-stimulated MΦ subsets. The production of IL-1β and IL-18 proteins was obviously dependent on the activation of caspase-1 pathway in M1-like MΦs. However, IL-1β production was caspase-1 dependent in M2-like MΦs whereas IL-18 production seems to be caspase-1 independent. This advises that in macrophages there are different regulatory mechanisms for IL-1β and IL-18 secretion. It was reported that there are examples where caspase-1 processing of IL-18 is not required where active IL-18 in caspase-1-deficient murine macrophages was released upon Fas ligand (FasL) stimulation (Tsutsui et al., 2000). Additionally, it was demonstrated that the signalling of IL-1β and IL-18 are related independently of NLRP3 or RIP3 (Dinarello et al., 2013a). It was suggested that even if caspase-1 inhibitor similarly inhibited the secretion of IL-1β and IL-18, they are seemingly regulated in a different way during infection.

# **Chapter 4: Endotoxin Tolerisation of MΦ subsets (Homo- and hetero-tolerisation)**



## 4.1 Introduction

Endotoxin tolerisation (ET) is a circumstance where cells go through a hypo-responsive state, unable to respond to further endotoxin-LPS challenge. ET has been studied *in vivo* and *in vitro* in both animals and humans (Biswas and Lopez-Collazo, 2009). Both host and pathogen would take benefits from ET and destructiveness alike; suppression of pro-inflammatory responsiveness leads to autoimmunity and cancer in the host (Foster and Medzhitov, 2009), while anti-inflammatory suppression is good for the pathogen. Gut mucosal MΦs are essential to ET because their differentiation and activation status determine whether the mucosal environment is harmful to the host tissue or pathogen. MΦs induced by ET exist by different mechanisms: presentation of anti-inflammatory cytokines (e.g. IL-10 and TGFβ), down-regulation of pattern recognition receptors (PRRs e.g. TLR4) or shedding of cytokine receptors and PRRs and /or induction of negative regulatory molecules, which have an inhibition role of TLR4 signal transduction, such as Tollip, Myd88s, SARM, IRAK-M and SIGIRR, reviewed by (Foey and Crean, 2013).

Mucosal macrophages MΦs have a dual functionality that determines tolerance to commensal organisms or immune response to entropathogens such as *E. coli* K12-LPS. The immune-suppression roles of M2 MΦs resemble to the feature of the endotoxin tolerance. To illustrate, M2 MΦs exhibit up-regulation of anti-inflammatory cytokine (e.g.IL-10) and show down-regulation of pro-inflammatory cytokines (e.g. TNFα) (Mantovani et al., 2005) however, it is an oversimplification to assign endotoxin tolerisation phenomenon to a specific MΦ subset because of the verities of MΦ phenotypes exhibited a consequence of differentiation, stimulation and tolerisation

(Cavaillon and Adib-Conquy, 2006a). Down-regulation of downstream immune responses in the tolerance to LPS is associated with reducing expression of TLR4 surface protein, Toll like receptor members, and /or absence of required co-receptor subunits MD-2 and CD14 (Cario and Podolsky, 2000a, Otte et al., 2004, Abreu et al., 2001). The endogenous suppressors such as Tollip, IRAK-M, SIGIRR and short version of MyD88 (MyD88s) are linked with down-regulation of the TLR4 receptor and LPS unresponsiveness (Biswas and Lopez-Collazo, 2009). Previously, tolerisation studies showed differential suppression between M1 (pro-inflammatory) and M2 (regulatory) MΦs in response to LPS of an oral pathogen, *Porphyromonas gingivalis*. It has been shown that the pro-inflammatory M1-like subset was refractory to tolerance induced by *P. gingivalis*, whereas, M2-like subset was sensitive to tolerance induced by *P. gingivalis* and suppressed the inflammatory cytokines (Foey and Crean, 2013). *E. coli* is an gut mucosal pathogen and *E. coli*-LPS is already understood to be able to induce ET in macrophages (Sun et al., 2014, Biswas and Lopez-Collazo, 2009).

Since IL-10, the anti-inflammatory cytokine, can suppress inflammatory responses of MΦs (Moore et al., 2001) it could play an important role in ET modulation. It is vital to investigate IL-10 functionality and signalling in MΦ subsets because the therapeutic value of IL-10 as well as its limited success in clinical trials (Fedorak et al., 2000, Tilg et al., 2002). It has been reported that IL-10 signaling is essential for the regulation of chronic and acute inflammation e.g., Crohn's disease and sepsis, respectively (El Kasmi et al., 2006). The signals of IL-10 are recognised by IL-10R $\alpha$  and IL-10R $\beta$  receptor complex prior to a signal cascade involving JAK1/TYK2 and STAT-3 (Foey et al., 2017, Fiorentino et al., 1991). STAT-3 is related with anti-inflammatory function, MΦ

stimulation inhibition, the expression of IL-10 (Benkhart et al., 2000, Donnelly et al., 1999).

The circumstance of cross-tolerisation has been defined between different microbial species, related PAMPs and the analogous PRRs, which might be involved in IBD, which, in addition to *E. coli*, is commonly determined in the gastrointestinal tract (GIT). It was suggested that cross-tolerisation is demonstrated between LPS, PGN and/or LTA (Biswas and Lopez-Collazo, 2009, Foey and Crean, 2013). It was interesting to investigate this process with respect to the distinct MΦ subsets profiles.

The main hypothesis of the research is to characterise the molecular mechanisms of ET in immune cells *in vitro* and to clarify the molecular basis of ET of other aspects' negative regulation of inflammation, Toll-like receptor TLR signalling, adaptor molecules and transcriptional factors and identification of novel molecules through genetic testing in addition to systems biology. The new findings and attempts to present an integrated and updated mechanistic view of ET might lead to improvement of the clinical significance with respect to several pathological conditions.

The aim of this study was to investigate the susceptibility of MΦ subsets to *E. coli* K12-LPS-tolerisation. Additionally, this investigation aimed to evaluate whether a range of PAMPs can induce cross-tolerance to K12-LPS and *vice versa* in THP-1 cells derived macrophage subsets M1 and M2.

The particular hypotheses to be verified in this investigation are:

**Hypothesis 1:** Endotoxin tolerisation by *E. coli* LPS (homo-tolerisation) can be induced in THP-1-derived MΦ subsets.

**Hypothesis 2:** PRRs and negative regulatory roles in ET induced in THP-1-derived MΦ subsets.

**Hypothesis 3:** anti-inflammatory cytokine, IL-10, modulate K12-LPS induced pro-inflammatory cytokine production in macrophage subsets.

**Hypothesis 4:** THP-1-derived MΦ subsets can induce cross-tolerance (Hetero-tolerisation) in response to different PAMPs include K12-LPS; TLR4 agonist, PGN; NOD2 agonist and LTA; TLR2 agonist.

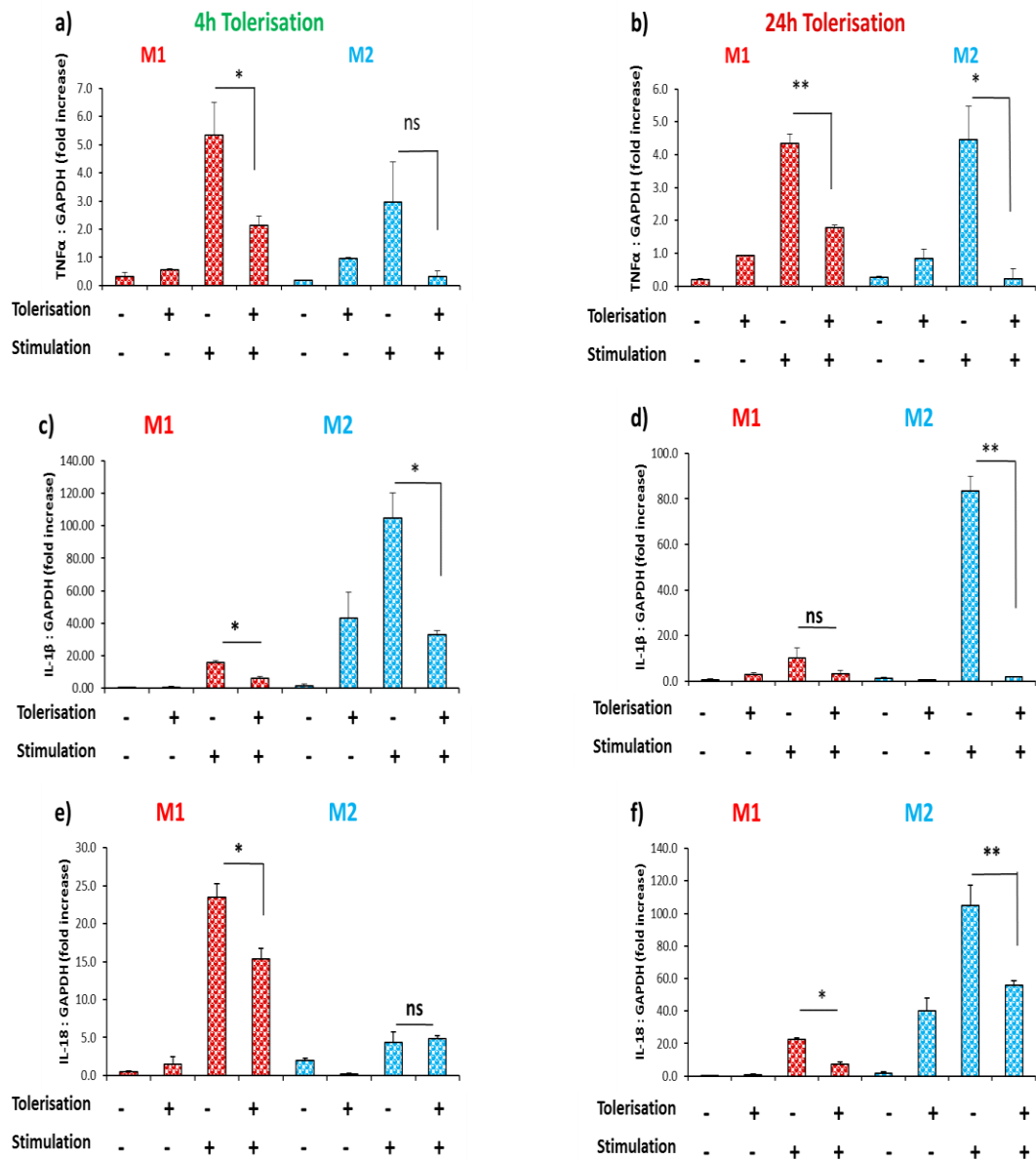
## **4.2 Results**

### **4.2.1 Expression of pro-inflammatory and anti-inflammatory cytokines mRNA in tolerised-MΦs.**

#### **4.2.1.1 Suppression of pro-inflammatory cytokines mRNA in endotoxin tolerised MΦs.**

In order to investigate ET molecular effects on MΦ subsets, gene expression of pro-inflammatory and anti-inflammatory cytokines have been investigated. Macrophage challenge with *Escherichia coli* (K12-LPS) differentially suppresses MΦ subset gene expression of cytokine of interest upon stimulation with the same pre-treatment challenges (homo-tolerisation). Gene expression of TNFα relative to GAPDH gene expression and comparing to LPS-stimulated MΦs, positive controls, was suppressed in both M1- and M2-like MΦs when pre-treated and then later challenged with K12-LPS in the two time points 4h and 24h. When pre-treated for 4h, TNFα gene expression level in M1 were significantly downregulated by 62%,  $P = 0.095$  and downregulated but insignificant in M2 and 83%,  $P = 0.15$  (Figure 4.2-1 a). Twenty-four hours of tolerisation showed similar level of TNFα gene expression reduction by M1 with 58%,  $P = 0.038$  and

higher and significant reduction by M2 with 93%,  $P = 0.012$  (Figure 4.2-1 b). IL-1 $\beta$  relative to GAPDH gene expression was also suppressed by both M1- and M2-like M $\Phi$ s in the two time points 4h and 24h. IL-1 $\beta$  gene expression level, when pre-treated for 24h were suppressed by 61%,  $P = 0.019$  in M1 and 64%,  $P = 0.045$  in M2 (Figure 4.2-1 c). However, 24h of tolerisation displayed insignificant level of reduction by M1 with 69%,  $P = 0.278$  and significant reduction by M2 with 97.5%,  $P = 0.006$  (Figure 4.2-1 d). The expression of pro-inflammatory IL-18 mRNA was downregulated significantly by M1-like M $\Phi$ s in 4h of tolerisation time 37%  $P = 0.041$  whereas there was no change observed by M2-like M $\Phi$ s (Figure 4.2-1 e). However, it was significant in both M $\Phi$  subsets after 24h tolerisation where M1 and M2 M $\Phi$ s showed 68% suppression,  $P = 0.032$  and 45%,  $P = 0.008$ , respectively (Figure 4.2-1 f).

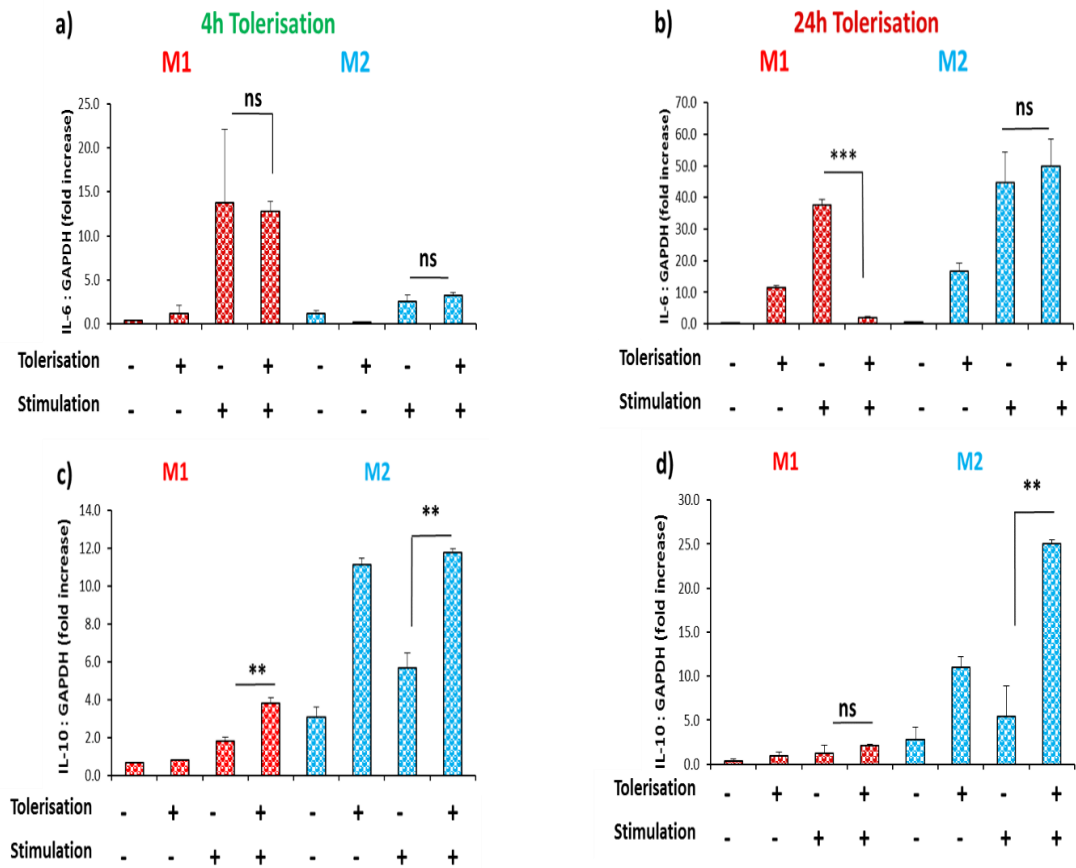


**Figure 4.2-1: Suppression of pro-inflammatory cytokine mRNA in endotoxin tolerised MΦs.**

M1 (Red) and M2 (Blue) MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 4h (a, c & e) and 24h (b, d & h) prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS, whereas (+) = LPS added for both pre-stimulated and stimulated cells. mRNA was extracted from cells to test the mRNA expression of TNFα (a, b), IL1β (c, d) and IL-18 (e, f) gene. Gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using  $2^{-\Delta\Delta Ct}$ . Data displayed is a representative experiment with duplicate samples of n= 2 replicate experiments. Significant effects compared to the positive control (-/+) are indicated as \* P < 0.05, \*\* P < 0.01 and ns= no significant.

#### **4.2.1.2 Differential expression of anti-inflammatory cytokines mRNA in endotoxin tolerised MΦs.**

IL-6 plays a vital role as anti-inflammatory cytokines via its ability to directly trigger the signal transducers and activators of transcription (STAT) factors STAT-1 and STAT-3, via the JAK/ STAT pathway (Hodge et al., 2005). There was no significant change in IL-6 gene expression comparing to positive controls (stimulation by K12-LPS without prior treatment) by M1 and M2 upon 4h pre-treatment. Nevertheless, after 24h pre-treatment, M1-like MΦs clearly suppressed IL-6 gene expression reduced by 95%,  $P=0.002$  but M2-like MΦ showed no significant change in IL-6 gene expression  $P=0.12$  (Figure 4.2-2 a & b). Interestingly, gene expression of anti-inflammatory; IL-10 showed significant upregulation by both MΦ subsets upon tolerisation in 4h time point. The increase level in IL-10 gene expression was 50% higher in M1- and M2-like MΦs,  $P=0.006$  and  $P=0.005$ , respectively (Figure 4.2-2 c). M1-like showed no significant change in IL-10 gene expression while M2-like MΦs did after 24h and the level of rise was 80%,  $P=0.006$  (Figure 4.2-2 d).



**Figure 4.2-2: Differential expression of anti-inflammatory cytokine mRNA in endotoxin tolerised MΦs.**

M1 (Red) and M2 (Blue) MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 4h (a & c) and 24h (b & d) prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. mRNA was extracted from cells to test the mRNA expression of IL-6 (a, b) and IL-10 (c, d) gene. Gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using  $2^{-\Delta\Delta Ct}$ . Data displayed is a representative experiment with duplicate samples of  $n=2$  replicate experiments. Significant effects compared to the positive control (-/+) are indicated as \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  and ns= no significant.

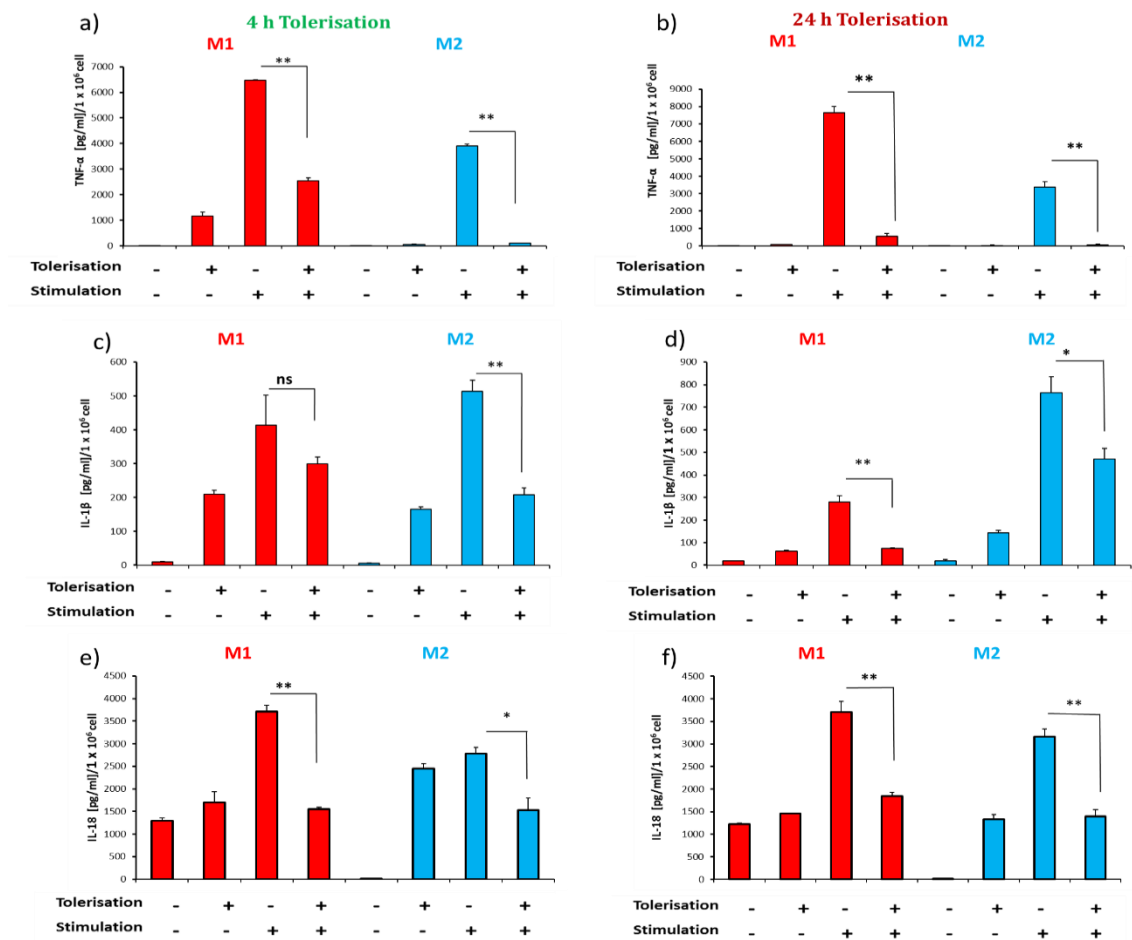


## **4.2.2 Effect of endotoxin tolerisation induced by *E. coli* LPS on cytokine secretion of macrophage subsets**

### **4.2.2.1 K12-LPS suppresses the pro-inflammatory cytokines secreted by MΦs.**

In order to compare with previous observed results in (Figure 4.2-1), 4h and 24h periods of pre-treatment with K12-LPS before stimulation with same challenges showed different levels of protein secretion of pro-inflammatory and anti-inflammatory cytokines. Macrophage challenge with *E. coli* K12-LPS differentially suppresses MΦ subset cytokine secretion upon stimulation with the same pre-treatment stimulus compared to positive controls, stimulation by K12-LPS without prior treatment (-/+). Four hours as earliest time point of tolerisation induction, and 24h as latest time point of tolerisation induction, of pre-treatment with K12-LPS before stimulation with same stimulus for 18h showed different levels of cytokine production (determined as optimal time course for production of all cytokines TNFα, IL-1β, IL-6 and IL-10, data not shown). Both M1- and M2-like MΦs significantly suppressed TNFα production when they pre-treated and then later challenged with K12-LPS in the two time points 4h and 24h. When pre-treated for 4h TNFα level produced by M1 and M2 were suppressed by 68%,  $P= 0.003$  and 95%,  $P= 0.003$ , respectively (Figure 4.2-3 a). Twenty-four hours of tolerisation showed greater reduction of TNFα secretion by M1-like MΦs with 92%,  $P= 0.002$  and M2 with 97%,  $P= 0.008$  (Figure 4.2-3 b). IL-1β cytokine production by tolerised M1-like MΦs was partially suppressed depending on time point of pre-treatment. M1-like MΦs failed to show any significant suppression of IL-1β after 4h, whereas it was strongly suppressed after 24h pre-treatment reduced by 80%,  $P= 0.007$ . M2-like MΦs presented a significant suppression after 4h and 24h pre-treatment reduced by 60%,  $P= 0.014$  and reduced by 55%,  $P= 0.056$ , respectively

(Figure 4.2-3 c & d). Both M1- and M2-like MΦs significantly suppressed IL-18 production when they pre-treated and then later challenged with K12-LPS in the two time points 4h and 24h. When pre-treated for 4h IL-18 level produced by M1- and M2-like MΦs were suppressed by 60%,  $P= 0.004$  and 42%,  $P= 0.035$ , respectively (Figure 4.2-3 e). Twenty-four hours of tolerisation showed similar reduction of IL-18 secretion by M1-like with 60%,  $P= 0.003$ , whereas M2-like showed higher reduction comparing to 4h results with 57%,  $P= 0.015$  (Figure 4.2-3 f).

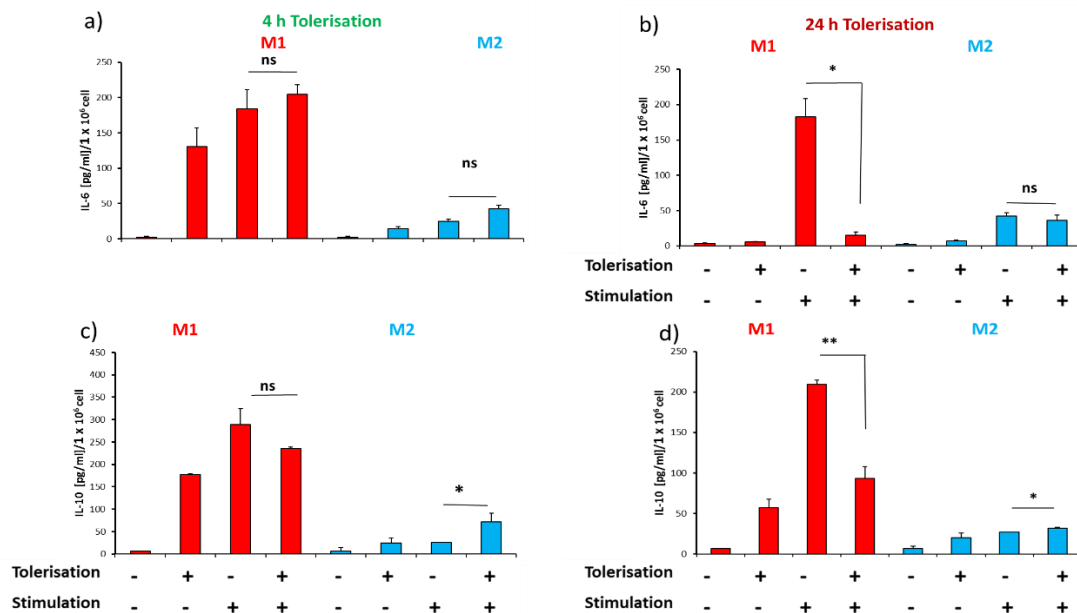


**Figure 4.2-3: Suppression of pro-inflammatory cytokine secretion by endotoxin tolerised MΦs.**

M1 (Red) and M2 (Blue) MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 4h (a, c & e) and 24h (b, d & f) prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. Cytokine production is expressed as the mean secretion  $\pm$  SD in pg/ml/ $10^6$  cells for TNF $\alpha$  (a & b), IL-1 $\beta$  (c & d) and IL-18 (e & f). Data showed represents triplicate samples for  $n= 3$  replicate experiments. Significant effects on suppression compared to the intolerised LPS control for the specified MΦ subset are indicated as \*  $p < 0.05$ , \*\*  $p < 0.01$  and ns= no significant.

#### 4.2.2.2 Anti-inflammatory cytokines by endotoxin tolerised MΦs.

There was no significant change in IL-6 cytokine production compared to positive controls, stimulation by K12-LPS without prior treatment (-/+), by both M1 and M2 upon 4h pre-treatment. However, after 24h pre-treatment, M1-like MΦs clearly suppressed K12-LPS induced IL-6 (reduction by 92%,  $P = 0.021$ ) but M2-like MΦs showed no change in IL-6 secretion (Figure 4.2-1 a & b). Pro-inflammatory M1-like MΦs showed no change in IL-10 production when they were pre-treated by K12-LPS for either 4h or 24h. On the other hand, anti-inflammatory M2-like MΦs showed up-regulation on IL-10 production upon 4h tolerisation with 66%,  $P = 0.035$  and 13%,  $P = 0.03$  upon 24h tolerisation (Figure 4.2-4 c & d).



**Figure 4.2-4: Differential sensitivity of MΦ-derived anti-inflammatory cytokines to endotoxin tolerisation.**

M1 (Red) and M2 (Blue) MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 4h (a & c) and 24h (b & d) prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. Cytokine production is expressed as the mean secretion  $\pm$  SD in pg/ml/10<sup>6</sup> cells for IL-6 (a & b) and IL-10 (c & d). Data showed represents triplicate samples for  $n = 3$  replicate experiments. Significant effects on suppression compared to the untolerised LPS control (-/+) for the specified MΦ subset are indicated as \*  $p < 0.05$ , \*\*  $p < 0.01$  and

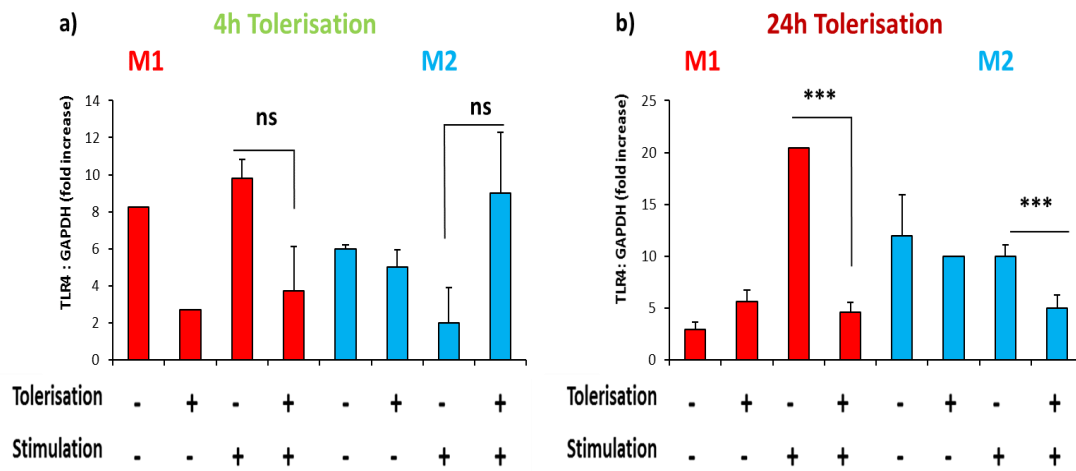
ns= no significant.

### **4.2.3 Differential gene expression of TLR4, TLR2 and NOD2 like receptors in endotoxin-tolerant MΦs.**

The previous data clearly revealed the outcome of ET on regulation of both pro-inflammatory and anti-inflammatory cytokines induced in MΦ subsets. As cell signalling controls the consequences of ET, the immunomodulation could occur via modulation of macrophage PRR expression including TLRs and NLRs. The data above leads to investigate the expression on TLRs and NLRs in endotoxin tolerised MΦ subsets.

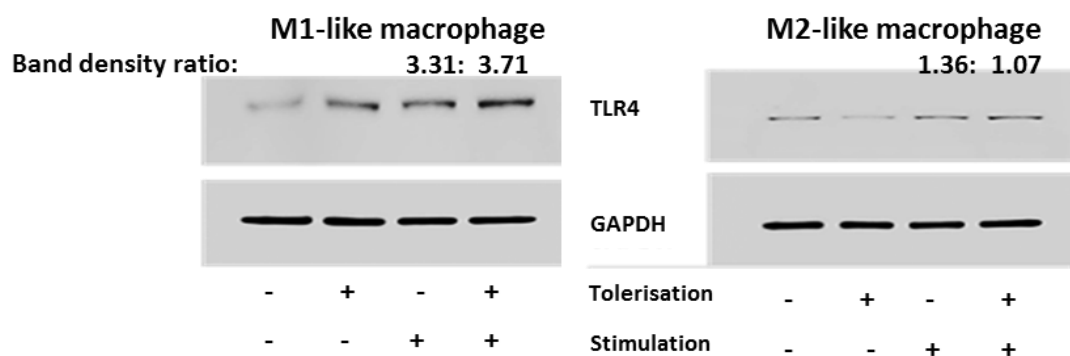
#### **4.2.3.1 TLR4**

As LPS is mainly recognised by TLR4, it was the first PRRs to be examined. Regulation of TLR4, LPS agonist, mRNA expression was observed in cells stimulated with K12-LPS. Upon 4h of tolerisation, gene expression of TLR4 showed no difference in level compared to positive controls by both M1-like and M2-like MΦs. After re-stimulation with the same LPS for additional 24h, the levels of mRNA expression of TLR4 was decreased markedly in M1- and M2-like MΦs compared with those from cells stimulated with LPS only once. Down-regulation was significant as 77%,  $P= 0.001$  and 50%,  $P< 0.001$  for M1 and M2, respectively (Figure 4.2-5 a & b). TLR4 protein after 24h tolerisation was also detected by Western blotting. Remarkably, M1-like MΦs presented a significant augmentation of TLR4 protein secretion, whereas M2-like showed slightly down regulation in TLR4 protein secretion comparing to positive control (Figure 4.2-6). The band density was measured for each band and the ratio to positive control was (3.31: 3.71) for M1-like and (1.36: 1.07) for M2-like MΦs.



**Figure 4.2-5: Endotoxin tolerisation effect on MΦ TLR4 gene expression.**

M1 (Red) and M2 (Blue) MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 4h (a) and 24h (b) prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. RNA was extracted from cells to test the mRNA expression of TLR4 gene. Gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using  $2^{-\Delta\Delta Ct}$ . Data displayed is a representative experiment with duplicate samples of n=2 replicate experiments. Significant effects compared to the LPS positive control (-/+) are indicated as \*\*\*  $P < 0.001$  and ns= no significant.

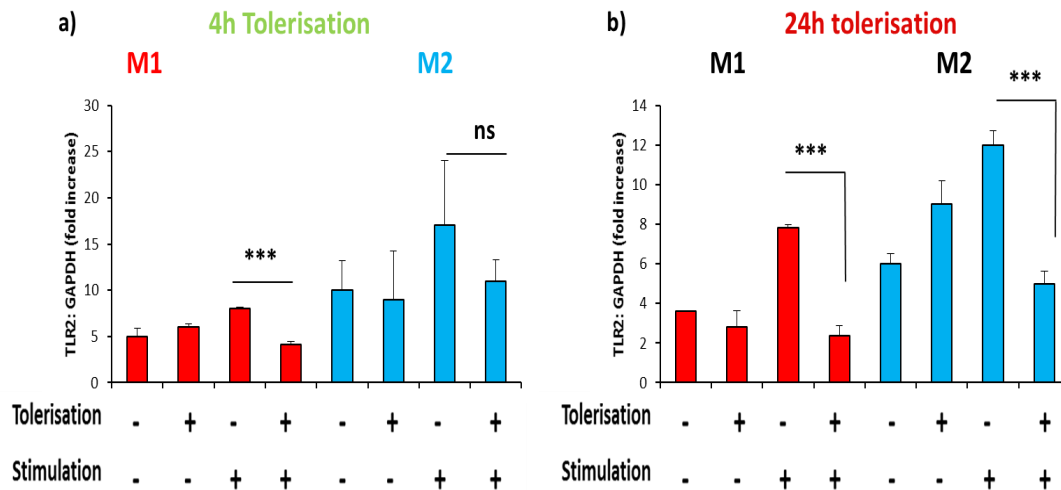


**Figure 4.2-6: ET differentially modulate TLR4 in distinct MΦ subsets.**

M1 and M2 MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 24h prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18hours (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. Protein activity of IRAK-M in K12-LPS tolerised MΦ subsets was detected by western blot. Western blot was used to explore IRAK-M protein expressions and levels of GAPDH were served as internal controls. The ratio of optical density compared to the negative control that was normalized to GAPDH controls. One representative result of three independent experiments is shown. Results was obtained in corporation with Barbara Durante.

#### 4.2.3.2 TLR2

In order to investigate the involvement of TLR2 in ET induced by K12-LPS, regulation of TLR2 gene expression was detected in MΦs stimulated with K12-LPS. Upon 4h of pre-treatment, mRNA expression of TLR2 showed strong suppression by M1-like 40%  $P < 0.001$  and no change in gene expression level comparing to positive controls by M2-like MΦs. After pre-stimulation with the same LPS for 24h, the levels of mRNA expression of TLR2 were decreased markedly in M1- and M2-like MΦs compared with those from cells stimulated with LPS stimulation. Down-regulation was significant as 75%,  $P = 0.001$  and 60%,  $P = 0.001$  for M1 and M2, respectively (Figure 4.2-7 a & b).

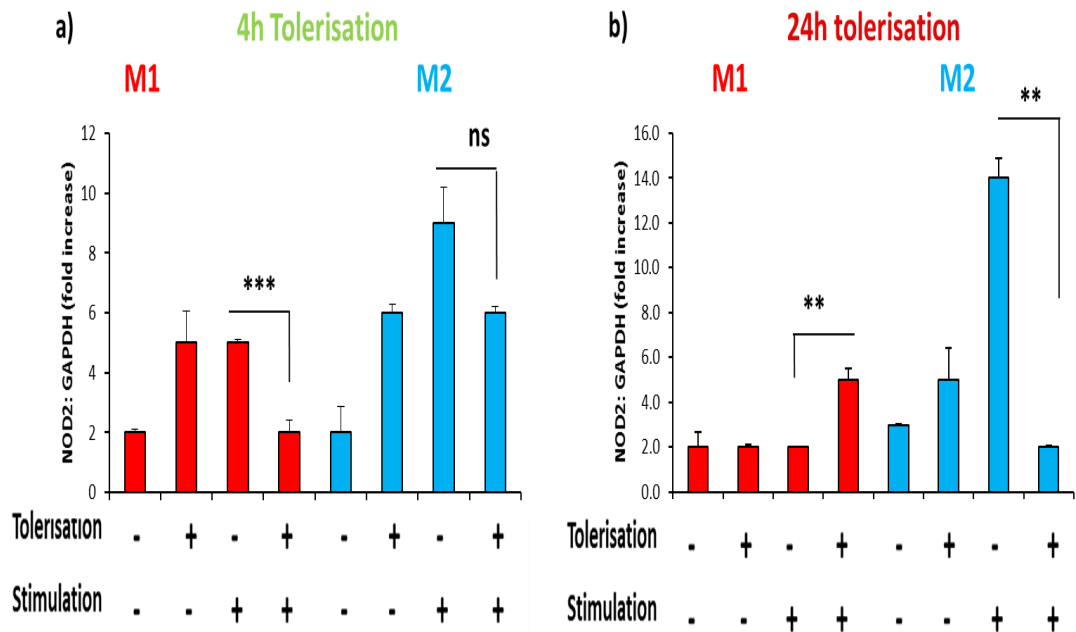


**Figure 4.2-7: Endotoxin tolerisation effect on MΦ TLR2 gene expression.**

M1 (Red) and M2 (Blue) MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 4h (a) and 24h (b) prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. RNA was extracted from cells to test the mRNA expression of TLR2 gene. Gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using  $2^{-\Delta\Delta Ct}$ . Data displayed is a representative experiment with duplicate samples of n= 2 replicate experiments. Significant effects compared to the positive control (-/+) are indicated as \*\*\* P < 0.001 and ns= no significant.

#### 4.2.3.3 NOD2

In addition to TLR2, the involvement of NOD2 in ET induced by K12-LPS was investigated. Role of NOD2 mRNA expression was noticed in MΦs challenged with K12-LPS. After 4h of tolerisation, gene expression of NOD2 to LPS stimulation was strongly suppressed by M1-like 60% P= 0.001 and no change in gene expression level comparing to positive controls by M2-like MΦs P= 0.142. Interestingly, after pre-treatment with the same LPS for 24h, the levels of mRNA expression of NOD2 to LPS stimulation were increased significantly in M1- 60%, P= 0.01. However, it was decreased markedly in M2-like MΦs compared with those from cells stimulated with LPS only once with 85%, P= 0.01 (Figure 4.2-8 a & b).



**Figure 4.2-8: Endotoxin tolerisation effect on MΦ NOD2 gene expression.**

M1 (Red) and M2 (Blue) MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 4h (a) and 24h (b) prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. RNA was extracted from cells to test the mRNA expression of NOD2 gene. Gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using  $2^{-\Delta\Delta Ct}$ . Data displayed is a representative experiment with duplicate samples of n= 2 replicate experiments. Significant effects compared to the LPS positive control (-/+ ) are indicated as \*\* P< 0.01 \*\*\* P< 0.001 and ns= no significant.

#### 4.2.4 Negative regulatory molecules are involved in endotoxin tolerisation mechanisms.

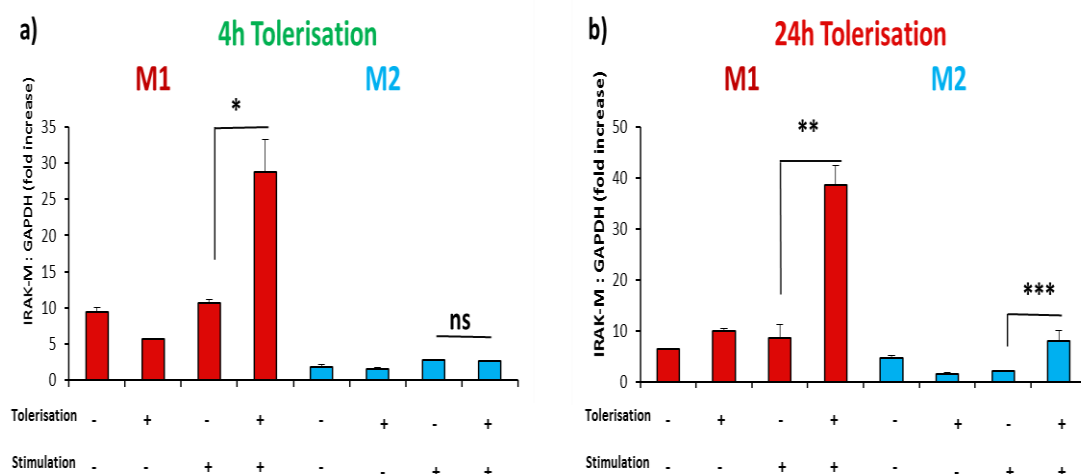
To find more about the mechanism of ET and considering if there is any difference in ET process between M1-like and M2-like MΦs, further study of the consequence of ET on selected negative regulatory molecules was done, either in level of mRNA expression by RT-PCR or protein secretion by WB. Negative regulatory molecules in this study include IRAK-M, Tollip and SIGIRR (mRNA and protein levels), SOCS3 and STAT-3 (mRNA level only). Endotoxin-tolerised MΦ subsets exhibited diverse levels of



negative regulatory gene expression and differential modulation of negative protein levels.

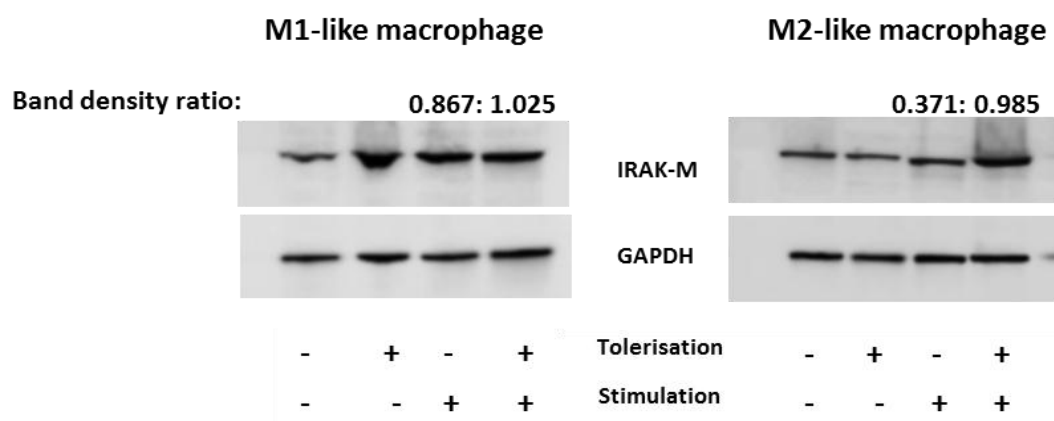
#### **4.2.4.1 IRAK-M**

Endotoxin-tolerised M1-like MΦs showed a significant up-regulation for IRAK-M mRNA in both tolerisation times, 4h and 24h, 62%,  $P = 0.04$  and 80%,  $P = 0.01$ . Although, IRAK-M mRNA levels did not change in M2-like MΦs in 4h tolerisation time point, it was significantly up regulated by 75% and  $P = 0.001$  in 24h tolerisation time point (Figure 4.2-9). Furthermore, IRAK-M protein, after 24hours of tolerisation, was also detected by Western blotting. Interestingly, M1-like MΦs failed to reflect similar results in IRAK-M protein level comparing to mRNA results. However, M2-like showed partly up-regulation in IRAK-M protein level (Figure 4.2-10). The band density was measured for each band and the ratio to LPS positive control (-/+ ) was (0.867: 1.025) for M1-like and (0.371: 0.985) for M2-like MΦs.



**Figure 4.2-9: ET regulates IRAK-M gene expression in MΦ subsets.**

M1 (red) and M2 (blue) MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 4h (a) and 24h (b) prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. RNA was extracted from cells to test the mRNA expression of IRAK-M gene. Gene expression is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak and Schmittgen, 2001) using  $2^{-\Delta\Delta Ct}$ . Data displayed is a representative experiment with duplicate samples of n= 2 replicate experiments. Significant effects compared to the positive control (+LPS) are indicated as \* P< 0.05, \*\* P< 0.01 \*\*\* P< 0.005 and ns= no significant.

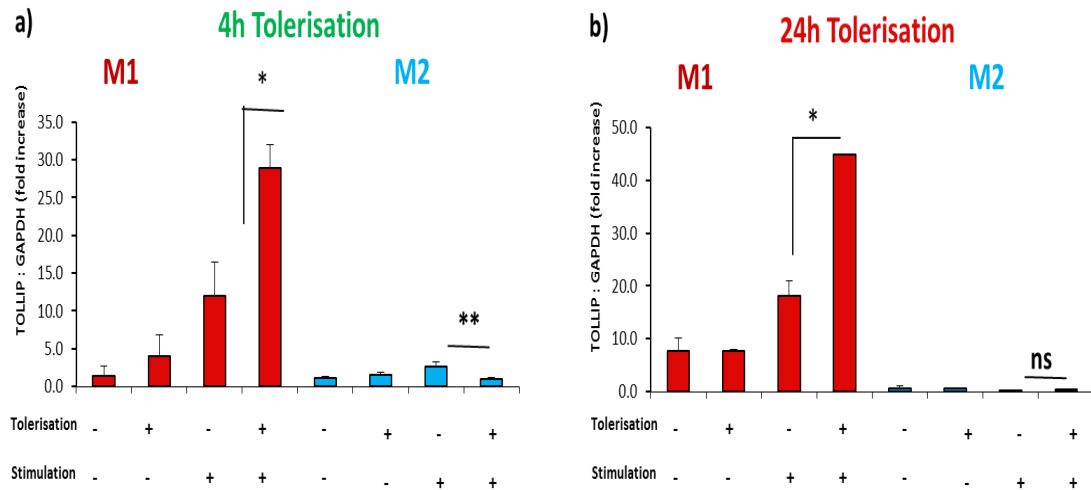


**Figure 4.2-10: ET differentially modulate IRAK-M in MΦ subsets.**

M1 and M2 MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 24h prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. Protein level of IRAK-M in K12-LPS tolerised MΦ subsets was detected by western blot. Western blot was used to explore IRAK-M protein expressions and levels of GAPDH were served as internal controls. The ratio of optical density compared to the negative control that was normalized to GAPDH controls. One representative result of three independent experiments is shown.

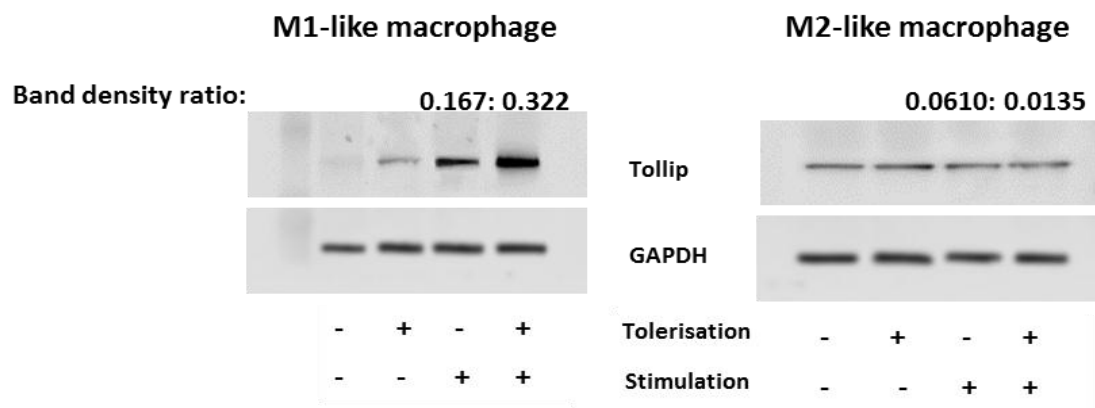
#### **4.2.4.2 Tollip**

A significant up-regulation for Tollip mRNA was shown by endotoxin-tolerised , pro-inflammatory, M1 MΦs in both tolerisation times, 4h and 24h, 47%,  $P= 0.02$  and 53%,  $P= 0.02$  respectively. Anti-inflammatory, M2 MΦs presented a downregulation of Tollip mRNA after 4h tolerisation by 40% and  $P= 0.01$  and no change after 24h tolerisation (Figure 4.2-11). Tollip protein secretion, upon 24h tolerisation, was also detected by Western blotting. Remarkably, M1-like MΦs presented a significant augmentation of Tollip protein level, whereas M2-like showed no change in Tollip protein level comparing to positive control (Figure 4.2-12). The band density was measured for each band and the ratio to LPS positive control (-/+) was (0.167: 0.322) for M1-like and (0.0610: 0.0135) for M2-like MΦs.



**Figure 4.2-11: ET regulates Tollip gene expression in MΦ subsets.**

M1 (red) and M2 (blue) MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 4h (a) and 24h (b) to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. RNA was extracted from cells to test the mRNA expression of Tollip gene. Gene expression is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak and Schmittgen, 2001) using  $2^{-\Delta\Delta Ct}$ . Data displayed is a representative experiment with duplicate samples of n= 2 replicate experiments. Significant effects compared to the positive control (+LPS) are indicated as \* P < 0.05, \*\* P < 0.01 and ns= no significant.

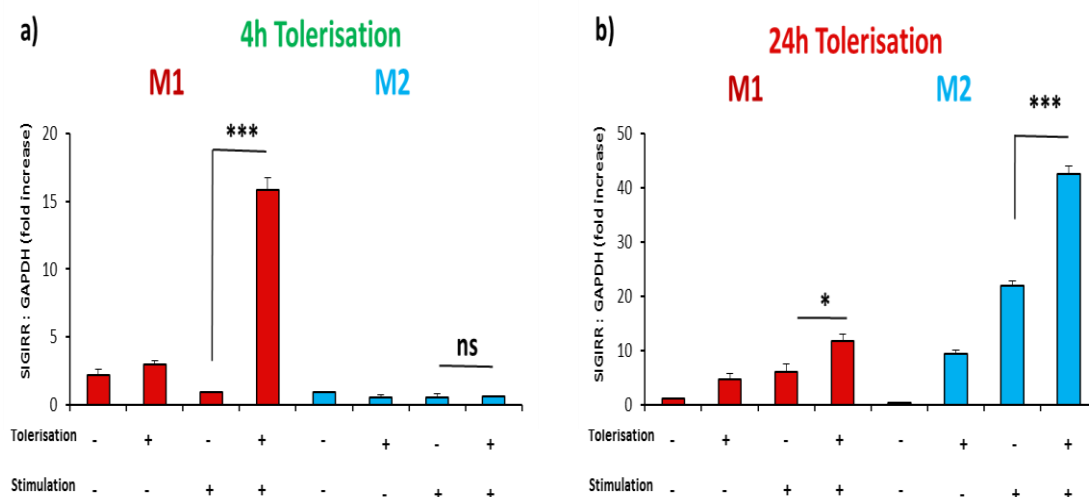


**Figure 4.2-12: ET differentially modulate Tollip in MΦ subsets.**

M1 and M2 MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 24h prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. Protein level of Tollip in K12-LPS tolerised MΦ subsets was detected by western blot. Western blot was used to determine Tollip protein expressions and levels of GAPDH were served as internal controls. The ratio of optical density compared to the negative control that was normalized to GAPDH controls. One representative result of three independent experiments is shown.

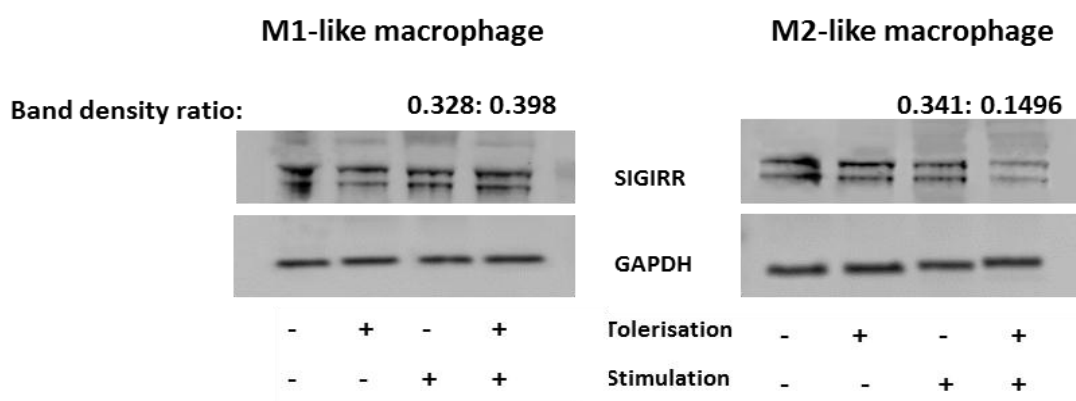
#### **4.2.4.3 SIGIRR**

SIGIRR can limit NF- $\kappa$ B-dependent luciferase expression as controlled by TLR4 and TLR9 (Zhao et al., 2012), was detected. M1-like MΦs presented a significant up-regulation for SIGIRR mRNA in both tolerisation times, 4h and 24h, 94%,  $P=0.001$  and 50%,  $P=0.03$ . While, SIGIRR mRNA levels did not change in M2-like MΦs at 4h tolerisation time point, it was significantly up regulated by 50%  $P=0.001$  at 24h tolerisation time point (Figure 4.2-13). Additionally, after 24h tolerisation, SIGIRR was also detected at protein level by Western blotting. M1-like MΦs presented a slight augmentation of SIGIRR protein level whereas, M2-like showed down regulation in SIGIRR protein level comparing to positive control (Figure 4.2-14). The band density was measured for each band and the ratio to LPS positive control (-/+) was (0.328: 0.398) for M1-like and (0.341: 0.1496) for M2-like MΦs.



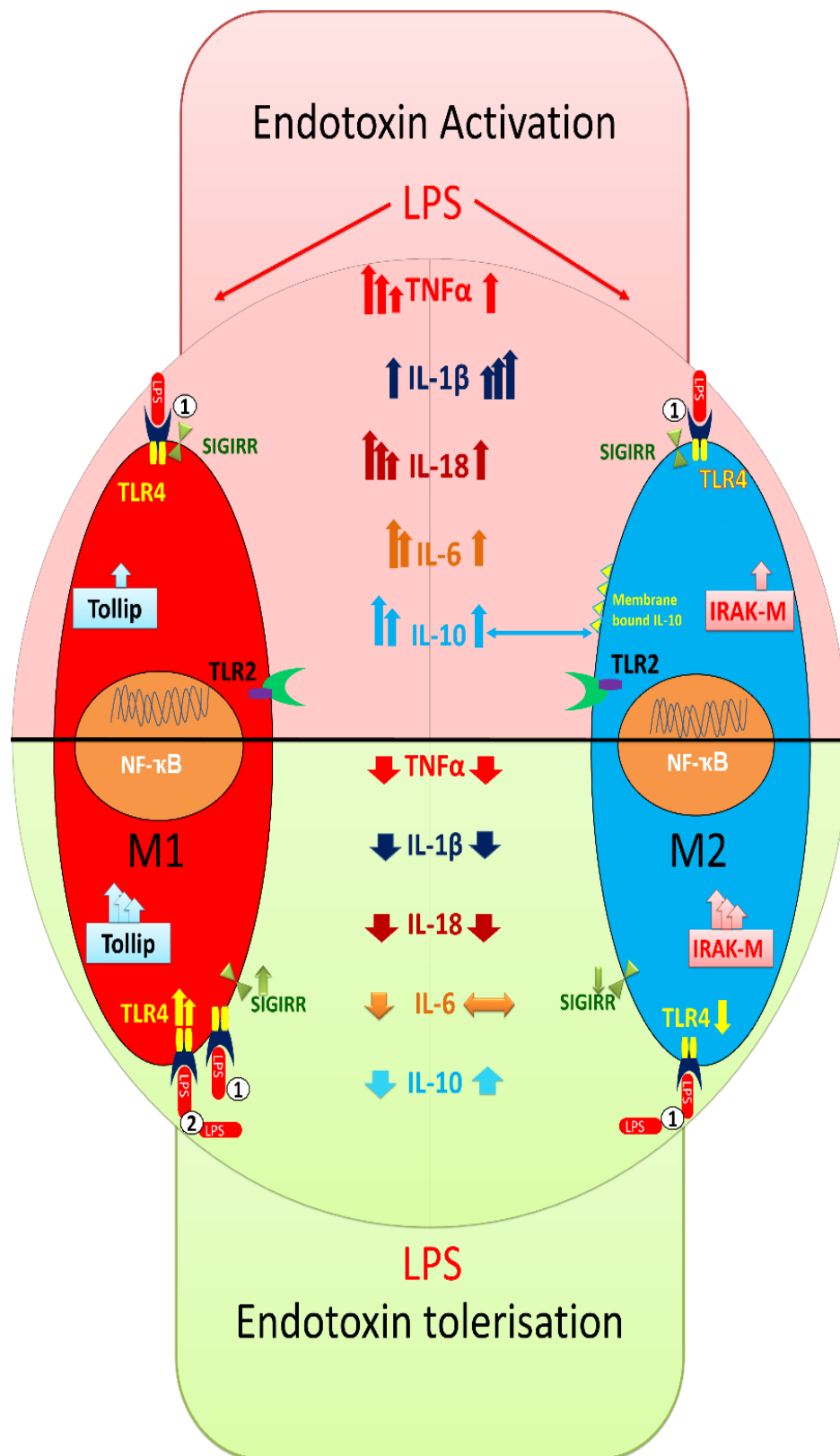
**Figure 4.2-13: ET regulates SIGIRR gene expression in MΦ subsets.**

M1 (red) and M2 (blue) MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 24h to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. RNA was extracted from cells to test the mRNA expression of SIGIRR gene. Gene expression is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak and Schmittgen, 2001) using  $2^{-\Delta\Delta Ct}$ . Data displayed is a representative experiment with duplicate samples of n=2 replicate experiments. Significant effects compared to the positive control (+LPS) are indicated as \* P<0.05, \*\*\* P<0.005 and ns= no significant.



**Figure 4.2-14: ET differentially modulate SIGIRR in MΦ subsets.**

M1 and M2 MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 24h prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. Protein level of SIGIRR K12-LPS tolerised MΦ subsets was detected by western blot. Western blot was used to determine SIGIRR protein expressions and levels of GAPDH were served as internal controls. The ratio of optical density compared to the negative control that was normalized to GAPDH controls. One representative result of three independent experiments is shown.



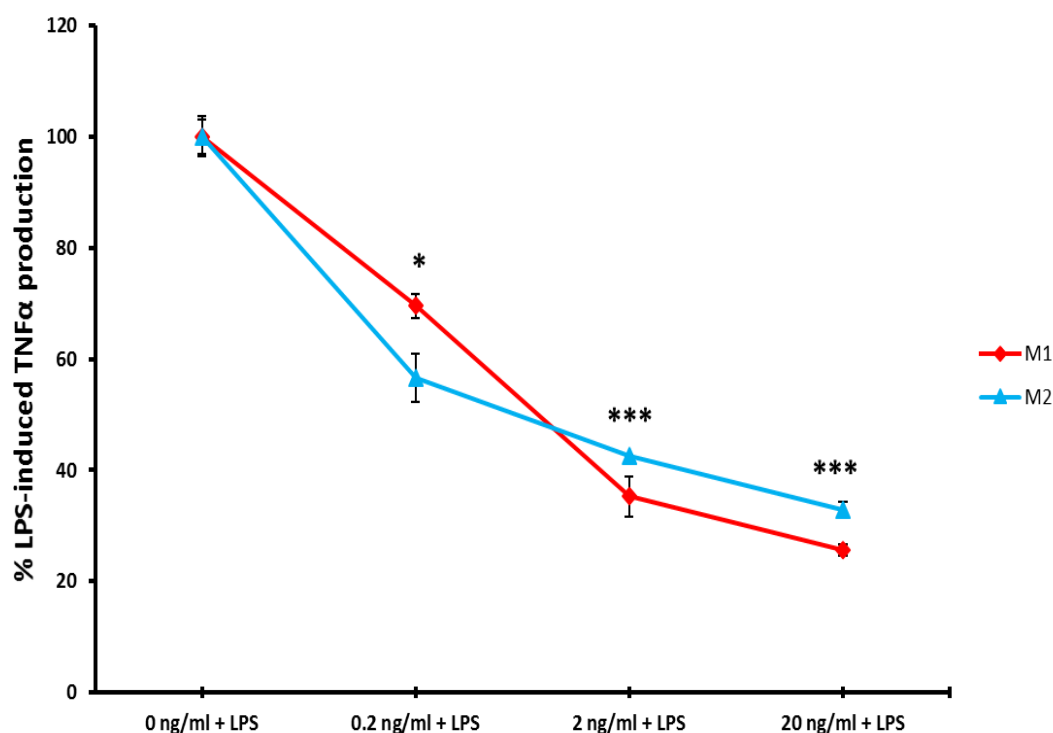
**Figure 4.2-15: endotoxin activation versus endotoxin tolerisation.**

This diagram showed the effect of LPS activation and LPS tolerisation on M1- and M2-like MΦs. It exhibits the different in cytokine production (TNF $\alpha$ , IL-1 $\beta$ , IL-18, IL-6 and IL-10), TLR2 and TLR4 expression and negative regulatory molecules Tollip, IRAK-M and SIGIRR. The diagram summarizes results obtained in this chapter.

#### **4.2.5 IL-10 modulates MΦ subsets response to K12-LPS**

MΦ subsets differentially produce secreted IL-10 and endogenous IL-10 activity upon K12-LPS challenge. To investigate the responsiveness of these MΦ subsets IL-10, another study was done to study the ability of IL-10 to suppress TNFα. Previous study has shown MΦ subsets to be differentially sensitive to IL-10 upon stimulation by LPS determined from the oral pathogen, *Porphyromonas gingivalis* (Foey et al., 2017). Interestingly, both MΦ subsets were sensitive to the anti-inflammatory effects of IL-10. Pro-inflammatory M1-like MΦ significantly suppressed K12-LPS-induced TNFα by 30%, 65% and 74% at concentrations of 0.2 ng/ml, 2 ng/ml and 20 ng/ml respectively. Anti-inflammatory M2-like MΦ also significantly suppressed K12-LPS-induced TNFα by 43%, 57% and 77% at concentrations of 0.2 ng/ml, 2 ng/ml and 20 ng/ml, respectively (Figure 4.2-16).





**Figure 4.2-16: Sensitivity of K12-LPS-induced MΦ TNFα to IL-10.**

M1 (Red) and M2 (Blue) MΦ subsets were stimulated with 100 ng/ml K12-LPS for 18h in the presence or absence of IL-10 at concentration range from 0 to 20 ng/ml. Anti-inflammatory effect of IL-10 on TNFα cytokine production is expressed as percentage of LPS-induced TNFα production originally represented as mean  $\pm$  SE in pg/ml. Data showed represents triplicate samples for n= 3 replicate experiments. Significant effects compared to K12-LPS control in the absence of IL- 10 (0 ng/ml) is indicated for MΦ subsets as \* P< 0.05 and \*\*\* P< 0.001.

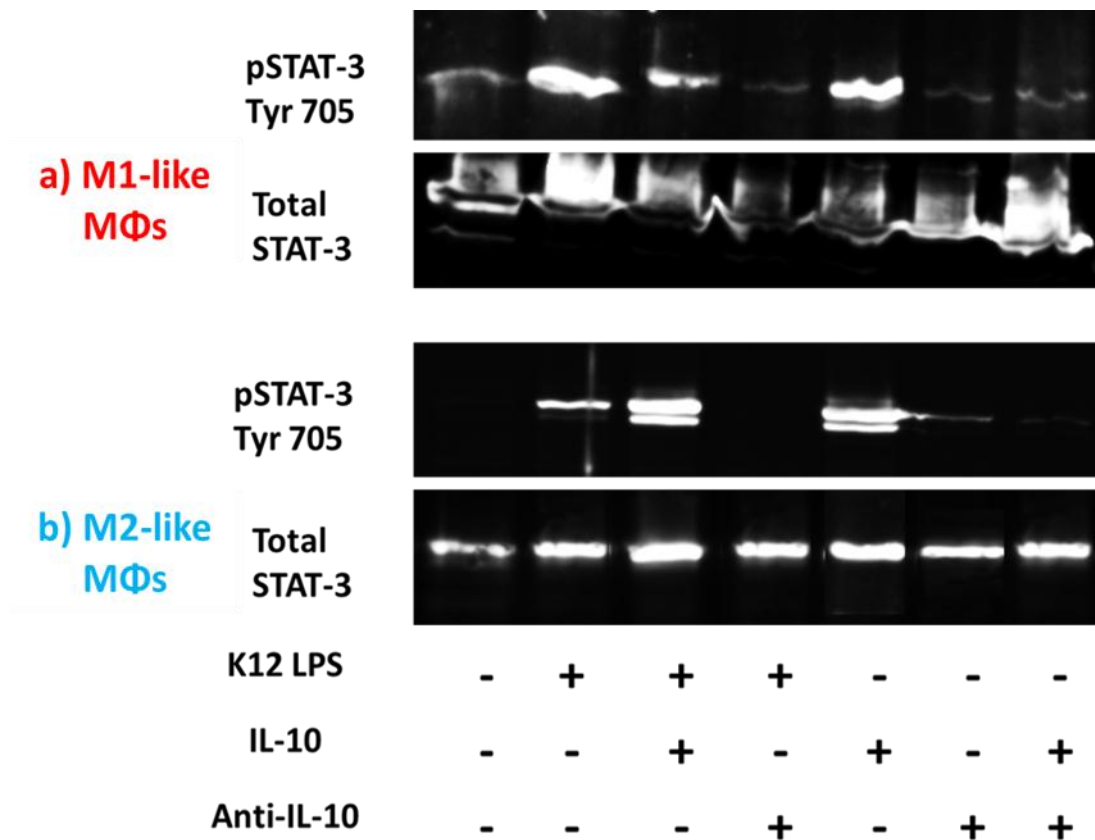
#### 4.2.6 STAT-3 activation in M1 and M2 MΦs.

To elucidate the mechanisms by which IL-10 inhibits LPS-induced TNFα production in MΦs, the signaling pathways activated in MΦs stimulated by IL-10 were explored.

##### 4.2.6.1 STAT-3 is differentially activated by K12-LPS and IL-10 in M1- and M2-like MΦs.

In order to understand the circumstances of the differential IL-10 profiles in response to K12-LPS and the similarity in responsiveness to IL-10 between M1- and M2-like MΦs, downstream of IL-10 receptor signalling have been investigated. STAT-3, downstream

of IL-10 receptor signalling, regulates anti-inflammatory responses. Therefore, STAT-3 activation might clarify this sensitivity to IL-10. Interestingly, Phospho- Western blot analysis showed activation of STAT-3 in both MΦ subsets but with some slight differences. To illustrate, although M1-like MΦs displayed clear bands of STAT-3 upon stimulation with either K12-LPS, IL-10 or both together, M1 failed to show two bands of STAT-3 in response to K12-LPS in the presence or absence of IL-10 (Figure 4.2-17,a). In contrast, K12-LPS activated STAT-3 in M2 MΦs increased by exogenous IL-10. M2-like MΦs showed one band in response to K12-LPS whereas two bands were displayed in response to K12-LPS present in combination with IL-10 as well as in response to exogenous IL-10. The K12-LPS-induced second band of phospho-STAT-3 was dependent on endogenous IL-10 neutralisation of which abolished STAT-3 activation (Figure 4.2-17, b).

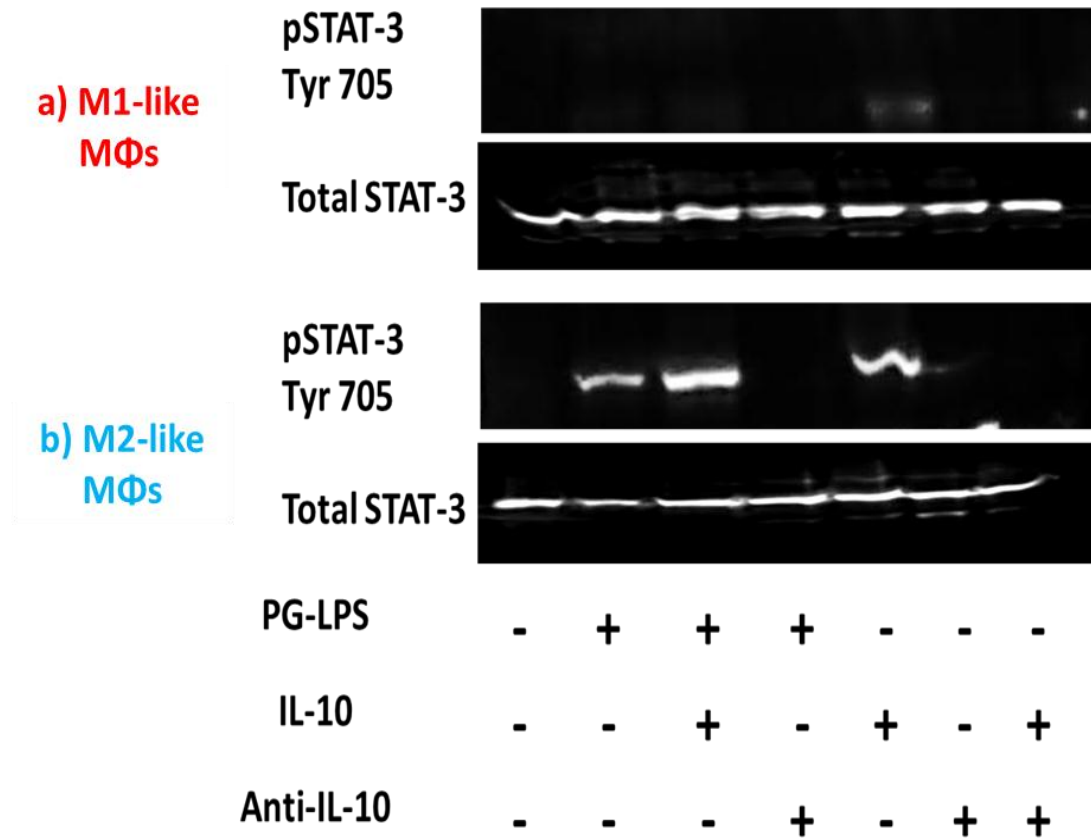


**Figure 4.2-17: STAT-3 differentially activated by K12-LPS and IL-10 in M1 and M2 MΦs.**

THP-1-derived M1 and M2 MΦs were plated out at  $1 \times 10^6$  cells/ml in a flat bottomed 12 well plate and treated with K12-LPS (100 ng/ml) in the presence or absence of exogenously added IL-10 (10 ng/ml) or neutralising anti-IL-10 antibody (10 mg/ml 9D7). After 30 minutes stimulation time, cell lysates were harvested. Western blot analysis of activated phospho-STAT-3 shows IL-10-mediated activation of STAT-3 in M1 (a) and M2 (b) MΦs. Loading controls are presented as total STAT-3 blots below the corresponding phospho-Westerns. Data displayed are representative of three replicate experiments.

#### **4.2.6.2        STAT-3 is differentially activated by PG-LPS and IL-10 in M1- and M2-like MΦs.**

In order to understand the differential sensitivity in responsiveness to IL-10 between M1- and M2-like MΦs, (Foey et al., 2017), downstream of IL-10 receptor signalling have been investigated. STAT-3, downstream of IL-10 receptor signalling, regulates anti-inflammatory responses. Therefore, STAT-3 activation might clarify this similarity sensitivity to IL-10. Interestingly, Phospho- Western blot analysis showed M1 MΦs failed to activate STAT-3 in response to PG-LPS in the presence or absence of IL-10 and a slightly activated STAT-3 when treated with IL-10 only (Figure 4.2-18, a). In contrast, PG-LPS obviously activated STAT-3 in M2 MΦs, increased by exogenous IL-10. The PG-LPS-induced phospho-STAT-3 was dependent on endogenous IL-10; neutralisation of which abolished STAT-3 activation (Figure 4.2-18, b).

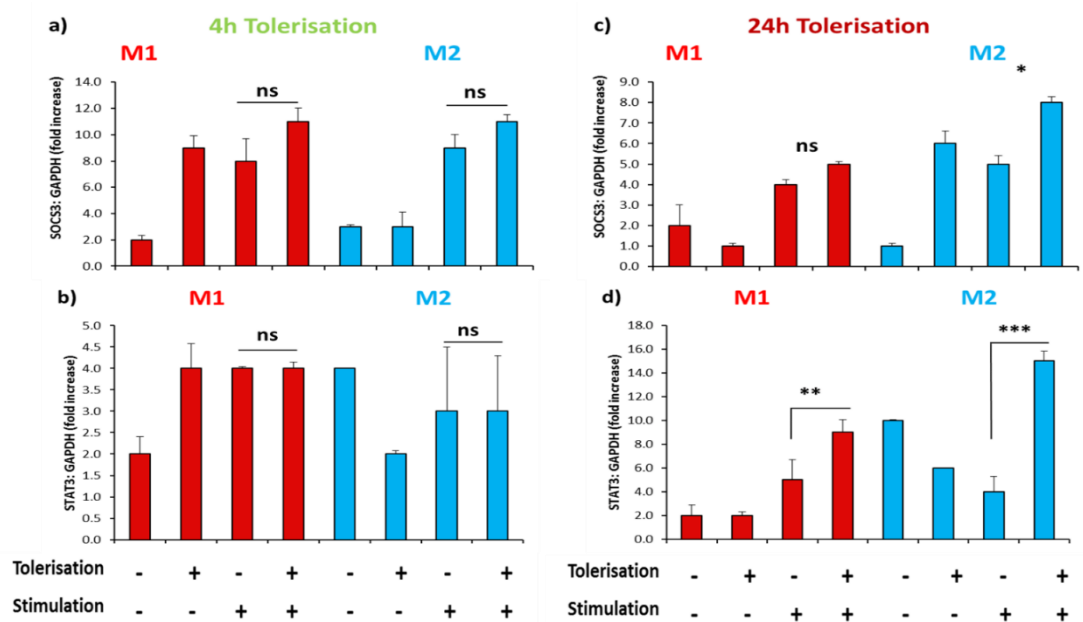


**Figure 4.2-18: STAT-3 is differentially activated by PG-LPS and IL-10 in M1 and M2 MΦs.**

THP-1-derived M1 and M2 MΦs were plated out at  $5 \times 10^6$  cells per well in a flat bottomed 12 well plate and treated with PG-LPS (100 ng/ml) in the presence or absence of exogenously added IL-10 (10 ng/ml) or neutralising anti-IL-10 antibody (10 mg/ml 9D7). After 30 minutes stimulation time, cell lysates were harvested. Western blot analysis of activated phospho-STAT-3 shows IL-10-mediated activation of STAT-3 in M1 (a) and M2 (b) MΦs. Loading controls are presented as total STAT-3 blots below the corresponding phospho-Westerns. Data displayed are representative of three replicate experiments.

#### 4.2.6.3 The expression of SOCS3 and STAT-3 genes in endotoxin-tolerised MΦs.

To obtain additional evidence that the JAK2-STAT-3 pathway functions in MΦ subsets, the effect of ET on SOCS3 mRNA expression was studied, which is inducible by STAT-3 activation. RT-PCR analysis revealed that SOCS3 mRNA expression in endotoxin-tolerised MΦs was upregulated only after long exposure to LPS followed by second challenge, 24h then 18h, by M2-like MΦs with 37%,  $P = 0.035$ . However, upon 24h tolerisation followed by 18h challenge with K12-LPS, ET showed up-regulation of mRNA of STAT-3 by 66%  $P = 0.004$  and 69%  $P = 0.0001$  in both MΦ subsets, M1- and M2-like, respectively (Figure 4.2-19).



**Figure 4.2-19: ET regulates SOCS3 and STAT-3 gene expression in MΦ subsets.**

M1 (red) and M2 (blue) MΦ subsets were pre-stimulated with 100 ng/ml K12-LPS for 4h (a & b) and 24h (c & d) to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. RNA was extracted from cells to test the mRNA expression of SOCS3 gene (a & c) and STAT-3 gene (b & d). Gene expression is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak and Schmittgen, 2001) using  $2^{-\Delta\Delta Ct}$ . Data displayed is a representative experiment with duplicate samples of  $n=2$  replicate experiments. Significant effects compared to the positive control (+LPS) are indicated as \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.005$  and ns = no significant.

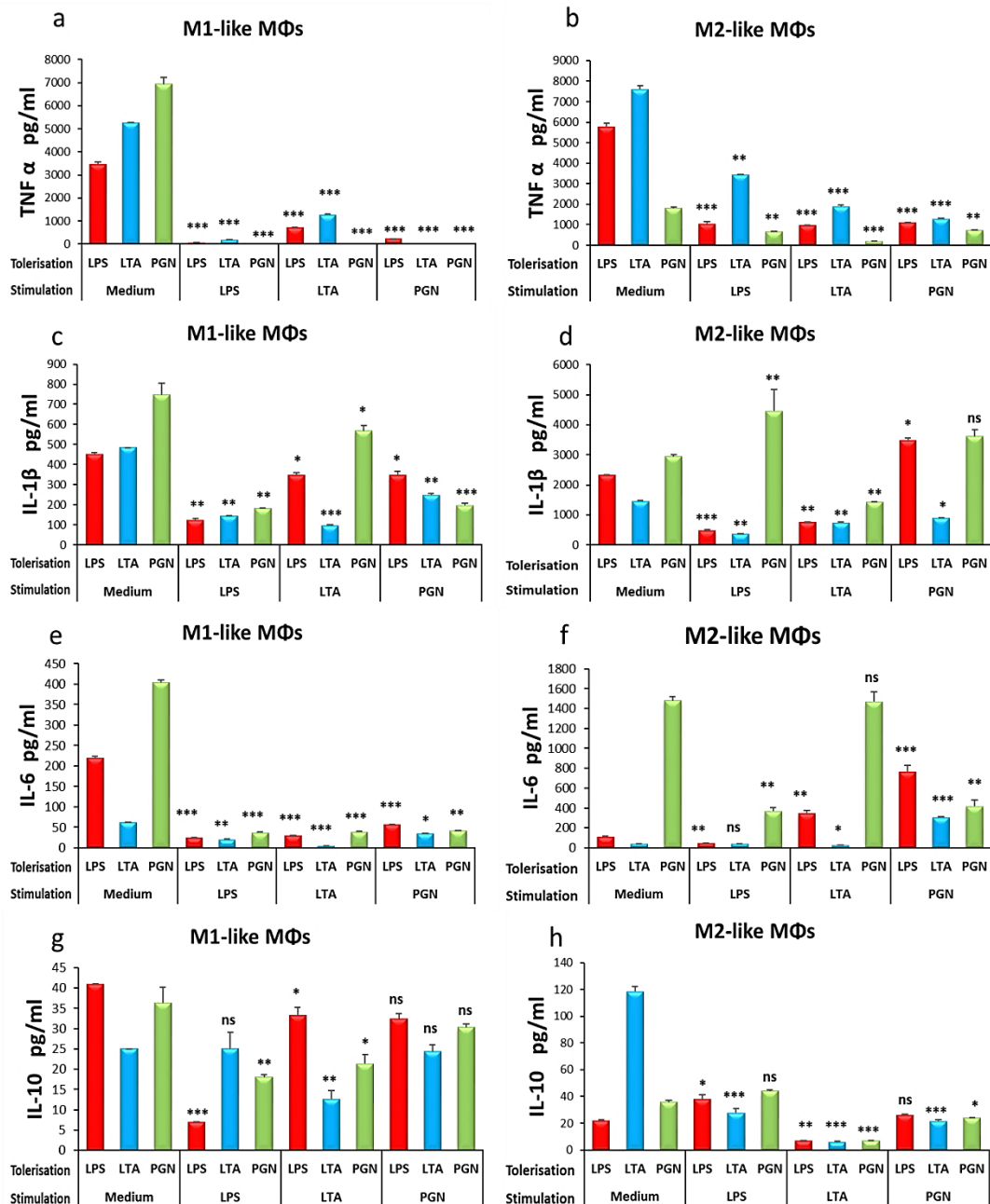
#### **4.2.7 MΦ subsets response to homo- and hetero-tolerisation induced by different PAMPs.**

Homo- and hetero-tolerisation were defined between different bacterial species, their PAMPs and the corresponding PRRs, which might take a part in the inflammatory process. Different PAMPs were used as either pre-stimulus or stimulus and these PAMPs include K12-LPS (TLR4), LTA (TLR2) and PGN (NOD2). The concentration of each agonist was chosen based on the ability to elicit comparable TNF $\alpha$  between the two MΦ subsets (Figures: Figure 3.2-1, Figure 3.2-4 and Figure 3.2-5). In general, tolerisation was obtained by both M1- and M2-like MΦs whatever the PAMPs were i.e. the suppression of TNF $\alpha$ , pro-inflammatory cytokine, was clear and significant in all cross-tolerisation protocol. TNF $\alpha$  suppression, compared to positive controls, was significantly more than 90% suppression by M1-like in all tolerisation protocols except two treatments. To illustrate, the case of LTA/LTA and LTA/LPS, the suppression was 75% and 80% respectively (Figure 4.2-20 a). TNF $\alpha$  induced by M2-like MΦs was significant. The significant TNF $\alpha$  suppression was in the case of LPS/LTA 55%, LPS/PGN 63% and PGN/PGN 60% less than positive controls (Figure 4.2-20 b) for P values see (Table 4.2-2). Upon pre-stimulation with LPS, the suppression of IL-1 $\beta$  by M1-like MΦs was 72% by LPS, 71% by LTA and 75% by PGN less than positive controls. Remarkably, there was significant suppression in IL-1 $\beta$  by M1-like MΦs in the case of LTA stimulation followed by LPS (23%) and PGN (24%). However, LTA homo-tolerisation resulted in very significant decreased in IL-1 $\beta$  by 80% less than positive control. Similarly, pre-stimulation with PGN suppressed IL-1 $\beta$  24% by LPS and 48% by LTA whereas, it was 74% less upon PGN homo-tolerisation compared to positive controls

(Figure 4.2-20 c). Additionally, upon pre-stimulation with LPS, the suppression of IL-1 $\beta$  by M2-like M $\Phi$ s was 80% by LPS, 75% by LTA and 34% by PGN less than positive controls. Similarly, pre-treated M2-like M $\Phi$ s by LTA resulted in significant decrease of IL-1 $\beta$  when stimulated with LPS 68%, LTA 48% and PGN 50%. Interestingly, LPS and PGN as stimulus increased IL-1 $\beta$  production by M2-like M $\Phi$ s upon pre-stimulation by PGN where the increase level was 33% by LPS and 19% by PGN. However, in the case of PGN/LTA, IL-1 $\beta$  was suppressed by 38% compared to positive controls (Figure 4.2-20 d). With regard to IL-6 production, there was some interesting differences i.e. M1-like M $\Phi$ s presented a significant reduction of IL-6 cytokine upon all homo- and hetero-tolerisation protocol (Figure 4.2-20 e). M1-like M $\Phi$ s presented significant suppression for IL-6, when pre-stimulated with LPS followed by LPS by 89%, LTA by 67% and PGN by 91%. M1-like M $\Phi$ s presented significant suppression for IL-6, when pre-treated with LTA followed by LPS by 86%, LTA by 93% and PGN by 90%. Likewise, M1-like M $\Phi$ s presented significant suppression for IL-6, when pre-treated with LPS followed by LPS by 74%, LTA by 45% and PGN by 90% (Figure 4.2-20 e). However, M2-like M $\Phi$ s presented significant suppression for IL-6, when pre-stimulated with LPS followed by LPS by 48% and PGN by 75%. In the case of LPS/LTA and LTA/PGN hetero-tolerisation, M2-like M $\Phi$ s displayed no change for IL6 by 0%. On the other hand, M2-like M $\Phi$ s significantly increased IL-6 in the case of LTA/LPS hetero-tolerisation by 67%, whereas IL-6 decreased by 35% upon LTA/LTA homo-tolerisation. Interestingly, IL-6 level significantly increased upon PGN/LPS and PGN/LTA hetero-tolerisation by 85% and 87%, respectively. Conversely, IL-6 decreased by 72% upon PGN/PGN homo-tolerisation (Figure 4.2-20 f). Remarkably, IL-10 profile presented by M $\Phi$  subsets was different. To illustrate, with M1-like M $\Phi$ s, LPS pre-treatment suppressed IL-10 upon LPS-stimulation by 82% and PGN-stimulation by 50%, whereas M1-like M $\Phi$ s showed



no change in IL-10 (1%) upon LPS/LTA hetero-tolerisation. LTA pre-treated M1-like MΦs suppressed IL-10 upon LPS-stimulation by 20%, LTA-stimulation by 48% and PGN-stimulation by 41%. However, IL-10 production showed no significant decrease upon PGN/LPS 19%, PGN/LTA 4% and PGN/PGN 16% (Figure 4.2-20 g). On the other hand, M2-like MΦs significantly increased IL-10 in the case of LPS/LPS homo-tolerisation by 43%, whereas IL-10 decreased by 76% upon LPS/LTA hetero-tolerisation and no significant change upon LPS/PGN. M2-like MΦs presented significant suppression for IL-10, when pre-stimulated with LTA followed by LPS by 68%, LTA by 95% and PGN by 80%. Furthermore, M2-like MΦs showed no significant change in IL-10 in the case of PGN/LPS hetero-tolerisation by 15%, whereas IL-10 decreased by 81% upon PGN/LTA hetero-tolerisation and 33% upon PGN homo-tolerisation (Figure 4.2-20 h) for P values see (Table 4.2-2).



**Figure 4.2-20: M1 and M2 MΦs subsets exhibit a differential cross-tolerisation of cytokine production to PAMPs.**

M1 (a, c, e & g) and M2 (b, d, f & h) MΦ subsets were pre-stimulated with either 100 ng/ml K12-LPS (red), 10 µg/ml LTA (blue) or 10 µg/ml PGN (green), in addition to medium only (control), for 24h (Tolerisation = pre-stimulation) prior further 18h (stimulation= second stimulation) with 100 ng/ml K12-LPS, 10 µg/ml LTA and /or 10 µg/ml PGN. Cytokines production are expressed as the mean secretion ± SD in pg/ml for TNFα (a & b), IL-1β (c & d), IL-6 (e & f) and IL-10 (g & h). Data showed represents triplicate samples for n= 3 replicate experiments. Significant effects compared to the positive control (+LPS) are indicated as \* P<0.05, \*\* P<0.01 \*\*\* P<0.005 and ns= no significant.

#### 4.2.8 Summary of chapter 4 results

**Table 4.2-1: Modulation on cytokines, PRRs and negative regulatory molecules by MΦ subsets upon endotoxin tolerisation .**

Macrophages		LPS tolerised- M1				LPS tolerised- M2			
		4hours		24hours		4hours		24hours	
		mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein
Cytokines	TNF α	↓	↓	↓	↓	↔	↓	↓	↓
	IL-1β	↓	↔	↔	↓	↓	↓	↓	↓
	IL-18	↓	↓	↓	↓	↔	↓	↓	↓
	IL-6	↔	↔	↓	↓	↔	↔	↔	↔
	IL-10	↑	↔	↔	↓	↑	↑	↑	↔
PRRs	TLR4	↔	N/A	↑	↑	↔	N/A	↓	↓
	TLR2	↓		↔	N/A	↓		↓	N/A
	NOD2	↓		↓	↔	↓			
Negative Regulator Molecules	IRAK-M	↑	N/A	↑	↔	↔	N/A	↑	↑
	Tollip	↑		↑	↑	↔		↑	↔
	SIGIRR	↑		↑	↑	↔		↑	↓
	SOCS3	↔		↔	N/A	↔		↑	N/A
	STAT3	↔		↑		↔		↑	

↑= increased, ↓= decreased, ↔= not significant.

**Table 4.2-2: Summary of cytokine profiles induced by MΦ subsets in response to homo- and hetero-tolerisation.**

			M1-like			M2-like		
Tolerant PAMPs	Stimulus	Cytokine induced	Change %		P value	Change %		P value
K12- LPS	LPS	TNFα	98%	↓	0.001 ***	81%	↓	0.0009 ***
	LTA		96%	↓	0.001 ***	55%	↓	0.008 **
	PGN		99%	↓	0.001 ***	63%	↓	0.0075 **
	LPS	IL-1β	72 %	↓	0.008 **	80%	↓	0.001 ***
	LTA		71%	↓	0.0074 **	75%	↓	0.0024 **
	PGN		75%	↓	0.010 **	34%	↑	0.01 **
	LPS	IL-6	89%	↓	0.001 ***	48%	↓	0.01 **
	LTA		67%	↓	0.009 **	0%	↔	0.18 ns
	PGN		91%	↓	0.001 ***	75%	↓	0.01 **
	LPS	IL-10	82%	↓	0.0005 ***	43%	↑	0.05 *
	LTA		0%	↔	1.2 ns	76%	↓	0.0014 ***
	PGN		50%	↓	0.01 **	18%	↑	0.12 ns
LTA	LPS	TNFα	80%	↓	0.001 ***	83%	↓	0.001 ***
	LTA		75%	↓	0.001 ***	75%	↓	0.0005 ***
	PGN		99%	↓	0.0003 ***	91%	↓	0.001 ***
	LPS	IL-1β	23%	↓	0.042 *	68%	↓	0.007 **
	LTA		80%	↓	0.001 ***	48%	↓	0.01 **
	PGN		24%	↓	0.033 *	50%	↓	0.01 **
	LPS	IL-6	86%	↓	0.0009 ***	67%	↑	0.0078 **
	LTA		93%	↓	0.001 ***	35%	↓	0.048 *
	PGN		90%	↓	0.001 ***	0%	↔	0.12 ns
	LPS	IL-10	20%	↓	0.048 *	68%	↓	0.01 **
	LTA		48%	↓	0.007 **	95%	↓	0.0002 ***
	PGN		41%	↓	0.042 *	80%	↓	0.001 ***
PGN	LPS	TNFα	93%	↓	0.001 ***	81%	↓	0.0025 ***
	LTA		99%	↓	0.001 ***	83%	↓	0.001 ***
	PGN		99%	↓	0.0003 ***	60%	↓	0.0032 **
	LPS	IL-1β	24%	↓	0.025 *	33%	↑	0.045 *
	LTA		48%	↓	0.009 **	38%	↓	0.05 *
	PGN		74%	↓	0.001 ***	19%	↑	0.124 ns
	LPS	IL-6	74%	↓	0.001 ***	85%	↑	0.0003 ***
	LTA		45%	↓	0.039 *	87%	↑	0.0004 ***
	PGN		90%	↓	0.003 **	72%	↓	0.0043 **
	LPS	IL-10	19%	↓	0.15 ns	15%	↑	0.33 ns
	LTA		4%	↓	0.20 ns	81%	↓	0.001 ***
	PGN		16%	↓	0.31 ns	33%	↓	0.045 *

↑= increase, ↓= decrease and ns = not significant

## Discussion

There are several conclusions from this investigation with respect to MΦ subsets behaviour responses to entropathogen *E. coli*. Firstly, ET induced by *E. coli*-K12-LPS failed to demonstrate a differential subset-specific response. K12-LPS tolerised M1- and M2-like MΦ subsets exhibited down-regulation of pro-inflammatory cytokines. However, with respect to anti-inflammatory cytokine secretion, M1-like MΦs showed down-regulation in IL-6 and IL-10 production, whereas M2-like MΦs showed no change in IL-16 and up-regulation in IL-10 level Secondly, the suppression of the pro-inflammatory cytokine of these MΦs appeared to be linked to the down-regulation of TLR4 and the differential up-regulation of the negative regulators, IRAK-M and Tollip. Thirdly, K12-LPS differentially induced IL-10 secretion and endogenous IL-10 activity in M1- and M2-like subsets. Furthermore, these subsets displayed similar sensitivity to IL-10-mediated suppression of K12-LPS induced TNFα, whereby M1 and M2 were both sensitive to IL-10.(Figure 4.2-16) This was in state in contrast to PG-LPS induced TNFα, where M2-like MΦs were suppressed by IL-10 and M-like MΦs were refractory to the suppressor added (Foey et al., 2017). In addition, although both subsets had similar sensitivity to IL-10, they presented STAT-3 activation. Finally, These MΦs displayed differential sensitivities to homo- and/or hetero-tolerance induced by *E. coli* and other PAMPs. Generally, there was a down-regulation in the cytokine production in both MΦs subsets in this study apart from IL-1β and IL-10, which were upregulated in the presence of PGN in M2-like MΦs.

#### **4.2.9 K12-LPS differentially suppresses M1 and M2 MΦs pro- and anti-inflammatory cytokines.**

Stimulation and pre-stimulation protocols investigate tolerisation by K12-LPS, allowing the study of signals via TLR4. In contrast to PG-LPS tolerisation studies in our laboratory (Foey and Crean, 2013), ET induced by *E. coli* K12-LPS failed to demonstrate a differential subset-specific response. These MΦs displayed differential sensitivities to tolerance induced by *E. coli*- derived bacterial PAMPs. K12-LPS tolerised M1- and M2-like MΦ subsets exhibited down-regulation of pro-inflammatory cytokines, whereas with respect to anti-inflammatory cytokine secretion; M2-like only showed increase in IL-10 production. Pro-inflammatory and anti-inflammatory MΦs performed an equal level of TNFα suppression in 24h, of pre-stimulation. The TNFα suppression in protein secretion was supported by the similar level of suppression shown in TNFα mRNA. Additionally, the suppression of IL-6 production in M1 MΦs might be associated with TNFα in some way. IL-10 mRNA produced by M1s is relatively stable in comparison to M2s according to results of time course of stimulated MΦ subsets (data not shown). This might clarify why M1 MΦs secreted higher level of IL-10 than M2 MΦs. Although the increase of gene expression of IL-6 and IL-10 by tolerised M2 MΦs is not significant but it could not be disregarded. ET is often related with over-secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β, which contribute to the deactivation of macrophages and the suppression of pro-inflammatory cytokine production (Biswas and Lopez-Collazo, 2009). Conversely, ET does not mean the total failure of all cytokines. Instead, it represents a selective reprogramming intended at reducing inflammatory damage (Melo et al., 2010). It might be very complicated to find out the reasons for differential cytokine production; however, it might be associated with distinct signaling pathways. The suppression of IL-1β might also result from the up-

regulation of these negative regulators because of the association of these negative regulators, TLR signal cascade and NF- $\kappa$ B activation. To illustrate, IL-1 $\beta$  production required activation of NF- $\kappa$ B (Kwon et al., 1995).

#### **4.2.10 Signalling mechanisms in ET in M1 and M2 M $\Phi$ s.**

*E. coli* LPS demonstrated different abilities to modulate expression levels of members of the TLR family. These results show that in response to repeated challenge of K12-LPS, the expression of TLR4 was down-regulated by M2-like M $\Phi$  subsets, which might reduce excessive signal transduction activated by PAMPs stimulations. The diverse expression profiles of cytokines secreted by LPS tolerised M $\Phi$ s could be related to the diverse regulatory mechanisms of the distinct expressions of TLR4. As a TLR4 agonist, K12-LPS, can activate MyD88-dependent and MyD88-independent pathways and present clear suppression in cytokine production in endotoxin-tolerised M $\Phi$ s, whilst TLR2 signals only through a MyD88-dependent pathways (Sun et al., 2014). In this current investigation, protein expression levels of TLR4 were explored, as M $\Phi$  subsets showed differential regulation in protein level of this receptor. Therefore, further studies are needed to explore the expression status of TLR4 in endotoxin tolerised M $\Phi$  subsets. It was supposed that there might be crosstalk between TLR4 and other receptors such as TLR2 and NOD2 pathways and some key signaling cascade downstream of these receptors could play a role in ET. Interestingly, there was a clear suppression of TLR2 gene expression in both tolerised M $\Phi$  subsets. These outcomes showed that in response to frequent LPS stimulation, TLR2 or TLR4 gene expression was suppressed, which might stop extreme signaling transduction activated by persistent bacteria stimulations. The expression of TLR2 and TLR4 proposed the different regulatory mechanisms, which might be in charge for the different cytokine

profiles secreted by K12-LPS tolerised MΦs. *E. coli* LPS (TLR4 agonist), triggers both MyD88-dependent and MyD88-independent pathways and suppresses cytokine secretion in endotoxin-tolerised cells, whereas TLR2 downstream activation goes only via MyD88-dependent pathway (Sun et al., 2014). It was established that pretreatment of THP-1 cells with bacterial lipoprotein suppressed TLR2 initiation in response to following challenge (Li et al., 2006). Although, LPS is a TLR4 agonist, LPS seems to play a role in NOD2 modulation in ET. Studies have proposed that concomitant stimulation of TLRs and NOD2 synergistically induces cytokines, although disagreement occurs about this matter (van Heel et al., 2005, de Jong et al., 2006). This study demonstrated in ET induced by MΦ subsets interesting results with regard to NOD2 gene expression. To illustrate, 24h after pre-treatment with the same LPS, the levels of mRNA expression of NOD2 was increased significantly in M1-like MΦs while it was decreased markedly in M2-like MΦs. In this research, the results prove the idea that NOD2 can specifically be modulated by TLR4 agonist, LPS. This might represent a vital mechanism via which overwhelming inflammatory responses to intestinal flora are suppressed. Nevertheless, these effects should be confirmed in future research on THP-1 derived MΦs. In addition, further studies on the specific molecular mechanism of the cross tolerance are also necessary.

The read out of cytokine suppression or up-regulation give us an idea about inflammatory profile and lead to investigate some tolerogenic mechanisms. Therefore, further investigations were done to compare and contrast the modulation in gene expression and protein level of negative regulators of endotoxin tolerised M1- and M2-like MΦs. Some negative regulators that have been studied; IRAK-M, Tollip and SIGIRR. IRAKs function is regulated through several mechanisms, such as the positive regulator IRAK4 and the negative regulator IRAK-M (Gottipati et al., 2008). IRAK-M is an effective



negative regulator of TLR signaling through MyD88, decreasing signaling through NF- $\kappa$ B and expression of pro-inflammatory such as TNF $\alpha$  and IL-1 $\beta$  (Kobayashi et al., 2002). It is assumed that the total expression levels of IRAK4 is suppressed in THP-1 cells and human monocytes in ET. Instead, ET reduced recruitment of IRAK4 to TLR4 and activation of IRAK4 (Xiong et al., 2011). Harada et al suggested that IRAK-M might inhibit the signaling between MyD88 and IRAK1 by binding to the complex, and then reduce the resulting cytokine production (Harada et al., 2006). IRAK-M is reported as; it is the only molecule whose expression has been confirmed in both mice and human ET models (Biswas and Lopez-Collazo, 2009). Tollip is associated with TLR expression and plays an inhibitory role in TLR-mediated cell activation. Zhang and Ghosh (2002) reported that Tollip associates directly with TLR2 and TLR4 and plays an inhibitory role in TLR-mediated cell activation, explaining that the inhibition by Tollip is mediated through its capability to suppress the activity of IL-1 receptor-associated kinase (IRAK) after TLR activation (Zhang and Ghosh, 2002). SIGIRR is a negative regulator involved in signal transduction of several receptors of the TLRs. Overexpression of SIGIRR can inhibit NF- $\kappa$ B-dependent luciferase expression mediated by TLR4 and TLR9 (Zhao et al., 2012). Interestingly, there was quite a difference in gene expression of these negative regulators between tolerised M1 M $\Phi$ s and M2 M $\Phi$ s. The significant up-regulation of expression of IRAK-M, Tollip and SIGIRR in tolerised M1 M $\Phi$ s that seemed to be related with the TNF $\alpha$  suppression it does not necessarily to be reflected in secreted protein i.e. fold differences observed in IRAK-M mRNA in M1-like M $\Phi$ s was less than fundamental mRNA expression after transcriptional alteration. ET differentially up-regulate negative regulatory molecules between M1 and M2 M $\Phi$ s subsets. In this study, Tollip and SIGIRR are mainly involved in ET induced in M1-like M $\Phi$ s whereas IRAK-M is associated with M2-like M $\Phi$ s. Additionally; more regulatory molecules were

evaluated in our investigation about ET induced by MΦ subsets such as STAT-3 and SOCS3. It was reported that STAT-3 plays an important role in IL-10-mediated inhibition of LPS-induced TNFα production (Nishiki et al., 2004). Macrophages induce SOCS3 by different mechanisms in response to microbial challenge and it could be the key of suppressing JAK/STAT signalling. IL-6 and STAT-3 phosphorylation are crucial to the pathology associated with inflammation like Crohn's disease, and raised SOCS3 expression proposes that SOCS3 may also have a regulatory role in these diseases (Crocker et al., 2008). Stimulated STAT-3 likewise encourages expression of SOCS3. SOCS3 in turn dismisses the JAK/STAT signalling cascade, creating a negative response loop that allows the MΦs to return to its basal (unstimulated) state (Babon et al., 2014). As mentioned above, the mutual association between SOCS-3 and STAT-3 would seem to control pro- or anti-inflammatory outcome of IL-6 and the polarisation of MΦs between M1 and M2 subsets. The upregulation in gene expression of STAT-3 in (Figure 4.2-19 d) might be explained why IL-6 production did not affect in ET induced by M1 and M2 MΦs. In this study, we demonstrated that although K12-LPS is able to induce similar sensitivity to tolerisation by both MΦ subsets, the mechanism of ET induction is different. Further study is needed to focus more in endogenous mechanisms of ET induced by MΦs and how these may be selectively related in distinct MΦ subsets induced in the case of harmful inflammatory response. Consequently, this research has described a differential, subset-dependent ET-induced up-regulation in separate negative regulators of TLR signal transduction pathways; where Tollip was increased in M1 MΦs and IRAK-M in M2s upon K12-LPS ET. Manipulation of the expression and binding activities of such regulators is likely to have a significant and discriminatory outcome on MΦ subset cytokine phenotype and therefore, regulation of ET may apply a reflective effect in the regulation of CD through

polarisation of MΦ subsets between anti-inflammatory, M2-like, and pro-inflammatory, M1-like, phenotypes.

#### **4.2.11 The differential induction and responsiveness to interleukin-10 displayed by M1 and M2 MΦs.**

The anti-inflammatory cytokine, IL-10, may be an important mediator of ET as it can discourage MΦs inflammatory responses (Moore et al., 2001). As discussed in the previous chapter (Figure 3.2-2), This study demonstrated that comparing to M1-like MΦs; M2-like MΦs differently produce IL-10 along with an endogenous cell-associated activity. The production of IL-10 is associated with functional dichotomy of MΦ subsets where M2 MΦs exhibited higher levels of both secreted and endogenous cell-associated IL-10 as result of K12-LPS stimulation. In contrast, M1 MΦs show lower level production as consequence of failure to exhibit an endogenous IL-10 activity. Similar finding was established previously in our laboratory with PG-LPS as well (Foey et al., 2017). It is well established that IL-10 plays important role in regulation the pro-inflammatory responses (Iyer and Cheng, 2012). IL-10 anti-inflammatory consequences is showed in various mechanisms such as TNFα activity down-regulation by encouraging TNF-R shedding or by modifying NF-κB signalling process producing TNFα cytokines (Foey et al., 2017). The hint of occurrence a level of cross-regulation and plasticity between M1 and M2 MΦ subsets leads to highlight the importance to study MΦ sensitivity to regulatory cytokines such as IL-10. In this study, we demonstrated that both M1 and M2 MΦs are sensitive to the anti-inflammatory effects of IL-10 in the presence of *E. coli* K12-LPS. Interestingly, previous studies demonstrated a differential sensitivity of MΦ subsets to IL-10, where M2 MΦs were sensitive and M1 MΦs were

refractory to the anti-inflammatory effects of IL-10 in the presence of PG-LPS (Foey et al., 2017).

The differential IL-10 responsiveness in the presence of PG-LPS was a consequence of activation of the downstream transcription factor, STAT-3 (Foey et al., 2017). However, the case was different when we investigated the IL-10 responsiveness in the presence of K12-LPS. To illustrate, both MΦ subsets displayed similar sensitivity to IL-10 in the presence of K12-LPS, which seemed to be as a result of STAT-3 activation despite the finding of that M2 MΦ showed a second band which exists in the presence of IL-10 (Figure 4.2-17). K12-LPS activates STAT-3 in M1 and M2 MΦs and PG-LPS activates STAT-3 in M2 MΦs only; this reaction is indirect via IL-10; neutralisation which abrogated the activation response. IL-10-dependency does not result from de novo IL-10 production because of the short stimulation time used in this experiment (30 mins) but it is likely to result from a pre-existing endogenous IL-10 activity detected for M2s. The increase of STAT-3 activation seems to be both IL-10-dependent in early responses and IL-10-independent later. IL-10 and STAT-3 are associated directly with anti-inflammatory and regulatory responses as well as indirectly through effects on MΦ polarisation. IL-10 and STAT-3 are essential key in MΦ plasticity and differentiation towards the M2 effector subset. STAT-3 is necessary for IL-10 signalling coupled with other pathways are essential for MΦ suppression. These mechanisms may include IL-10-inducible STAT3-regulated gene expression of SOCS3 that negatively control IL-10 signalling (Lang et al., 2003, Foey et al., 2017). In addition to its role in regulation of anti-inflammatory responses, SOCS3 plays another role in M2 to M1 switch by driving MΦ plasticity to pro-inflammatory phenotype (Liu et al., 2008). This ability of adjusting plasticity between M1 and M2 subsets is helping to detect suitable immune response

either pro-inflammatory or anti-inflammatory and this might be beneficial in the treatment of inflammatory diseases (Foey and Crean, 2013).

#### **4.2.12 Homo- and hetero-tolerisation induced by different PAMPs**

The different mechanisms of ET have been mostly studied with respect to LPS-TLR4-mediated ET or Gram-negative sepsis (Biswas and Lopez-Collazo, 2009). Nevertheless, ET is derived across also upon pre-stimulation by other TLR ligands and in Gram positive (i.e. LPS negative) sepsis. Tolerance to an endotoxin induced by previous introduction to a dissimilar endotoxin is mentioned as hetero-tolerance or cross-tolerance. For example, earlier exposure to TLR2 ligands or NOD2 ligand, such as Lipoteichoic acid (LTA) and peptidoglycan (PGN) followed by second exposure to K12-LPS is presented as cross-tolerance and *vice versa* (Figure 4.2-20 and Table 4.2-2).

In this investigation, it has been demonstrated that M1-like MΦ could induce a clear suppression in most pro- and anti-inflammatory cytokines produced in homo- and/or hetero-tolerisation. Interestingly, it was only IL-6 that was upregulated upon PGN/LTA tolerisation as well as IL-10 that displayed no significant change upon PGN/LPS and PNG/PGN tolerisation. M2-like MΦs showed similar suppression attitude apart from interesting deferent results especially when PGN is present. Remarkably, IL-1β was upregulated upon stimulation or tolerisation M2-like MΦs by PGN i.e. (LPS/PGN, PGN/LPS and PGN/PGN tolerisation). Wan et al., presented in his study that purified cell wall PGN was able to induce IL-1β in human cells (Wang et al., 2000). This upregulation of IL-1β in the present of PGN need more investigation especially in M2-like MΦs where they showed unexpected IL-1β production as obtained before in (Figure 3.2-11) and later in (Figure 5.2-10). It was established that NOD2 activation resulted in cross-tolerance to TLR4-dependent production of TNFα, the dominant

cytokine mediating inflammation in Crohn's disease (Kim et al., 2008). Although Kim et al presented in his investigation that the effect of NOD2 tolerisation is specific for TLR4 stimulation and TLR2 ligands was not affected (Kim et al., 2008), we established that cross-tolerisation between LTA and PGN can be induced in both MΦ subsets. We found that TNFα, IL-1β and IL-10 was downregulated by both M1- and M2-like MΦs upon PGN/LTA and LTA/PGN tolerisation. However, PGN failed to suppress IL-6 production by MΦ subsets upon LTA stimulation where M1-like exhibited a significant upregulation with 64% and M2-like showed no significant change in the level of IL-6. There were no cross-tolerisation found in some studies between MDP and TLR2 in human monocytes or TLR4 signaling in the secretion of IL-6 (Kullberg et al., 2008). It was also reported in different study that there was no change in IL-6 production in response to the several stimuli (Kullberg et al., 2008).

This study gave additional characterisation for M1- and M2-like MΦ subsets with respect to K12-LPS-induced activation/ tolerogenic conditions, which showed the discrepancy of ET induced by K12-LPS and PG-LPS. Furthermore, the similar sensitivity to IL-10-mediated suppression observed that both MΦ subsets were sensitive to IL-10 suppression in resent of K12-LPS whereas the pro-inflammatory pathological subset, M1 was less sensitive to IL-10 suppression in present of PG-LPS. This would suggest that to obtain more understanding of ET mechanisms, LPS types and characteristics need to be considered. The main objective is to improve the methods to induce a selective MΦ subset suppression's manipulation. Therefore, the balance of activation and suppression between MΦ subsets may contribute to adaptable future beneficial method for the regulator of such destructive inflammatory bowel pathologies that can

only be occupied upon full thoughtful of the mechanisms driving such relapsing events  
characteristic

# **Chapter 5: Inflammatory responses in an epithelial cell & macrophage co-culture model**



## 5.1 Introduction

The connection between epithelial cells and macrophages is vital in mucosal immune processes. Intestinal homeostasis is dictated by epithelial tissue, which permits the gut to actively sample pathogens, resident bacteria and other antigens derived from digester such as CD28 and cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) (Read et al., 2000). The mucosal membrane is comprised of mucus, and it envelops the epithelial tissue, functioning as a defensive barrier that restricts pathogen access (Kenny et al., 2008). The crucial element in this functionality is mucosal macrophages that regulate suitable immune reactions to pathogens (Vajdy, 2008).

In the GIT, epithelial cells have a range of roles, including forming the physical boundary to form the distinction between the external environment of microbiota and food antigens from the internal environment, where the cells of the lamina propria such as macrophages are resident. Elicited macrophage reactions are reliant upon the tissue situation and the subsequent cell subsets, with M1 subsets are representing inflammatory macrophages whereas, homeostatic macrophages are represented by M2 subset (Mantovani et al., 2007). The particular aetiology of IBD, the chronic inflammatory condition of the gastrointestinal tract that incorporates Crohn's disease (CD) and Ulcerative Colitis (UC), remains a matter of debate. This said, an awareness of the pathophysiology of IBD has developed, and the conventional elements of these conditions present themselves in a range of research within *in vivo* and *in vitro* concepts. As CD is associated with cell mediated immunity more relate with Th1 whereas UC is associated with humoral immunity which is related to Th2 and the cytokine that drive these responses are more associated with M1 and M2 subsets i.e.

the actual macrophage subset allows a potential to study IBD through this delineation process. Therefore, M1 macrophage could be important in studying cell mediated immune pathology associated with IBD (CD), whereas M2 macrophages might be more appropriate to study humoral responses which is more related to UC.

To resemble the *in vivo* situation, *in vitro* cell models of the gut utilising an array of varying cell forms was implemented to explore the PAMPs immunomodulation of pro-inflammatory cytokines on each specific cell type in co-culture model and the barrier cell function and cytokine generation profile for epithelial-like cells. Expression of tight junction proteins is required for the creation of an epithelial barrier, integrity, and polarity. Epithelial cells undertake a range of different progressions to devise a monolayer with a regulated selectively porous membrane with a trans-epithelial restriction that can be quantified. Epithelial cells when linked with other cell lines comprise a model representing gut physiology, should react to environmental aspects, including cytokines and inflammatory molecules. Of these, a good concept for intestinal epithelial cells is Caco2, and THP-1 human monocytic cell line for monocyte derived M1-like and M2-like macrophage cell subsets. As such, these two cells were utilised to devise a model, where epithelial cells (Caco2) combined with M1-like MΦs to model inflammatory gut physiology or with M2-like MΦs as a conventional homeostasis model. Caco2 cells upon reaching confluence, express attributes of enterocytic variation and purpose, with aspects including microvilli, large vacuolated mitochondria, and smooth and rough endoplasmic reticulum (Lee et al., 2009). Lo et al. (2004) asserted that transwell based Caco2/Raji B cell culture model introduced differentiating enterocytes into M (microfold) cells (Lo et al., 2004). This indicated that the M-like Caco2 cells had in common aspects with M cells in FAE. Bacterial transport

of *Salmonella* – some alive, some dead – was additionally investigated via such concepts so as to verify the purpose of the concept for exploring transcytosis and cell signalling pathways in M-like cells (Martinez-Argudo and Jepson, 2008). Nevertheless, Caco2 cells are polyclonal and this high diversity has led to the necessity for set procedures, such as in relation to the passage number and the time of usage post-seeding for adherence. Furthermore, the plan of Caco2 was co-cultured in relation to the other cell forms, including PBMCs and macrophages. Parlesak et al. (2004) asserted that the co-culturing of Caco2/PBMCs modulate the cytokine kinetics within a challenge with bacteria (non-pathogenic *Escherichia coli*) within a compartmentalized co-culture concept by Caco2. Additional bacteria was applied apically to the Caco-2 cell layer, the generation of TNF $\alpha$ , IL-12, IL-1 $\beta$ , IL-8, IL-6, IL-10, and TGF- $\beta$  was significantly reduced in relation to the bacterial stimulation of leucocytes underneath the Caco2 cells. The modulation of cytokine expression by Caco2 cells helps the model of leucocyte (neutrophils, mast cells, NK, T cells, B cells etc.)-epithelial cell crosstalk functions in modulating cytokine reactions in the gut mucosa (Parlesak et al., 2004). Results of a similar nature were identified by (Haller et al., 2000) when Caco2 co-cultured with human blood leucocytes and profiles of TNF- $\alpha$ , IL-1 $\beta$ , IL-8, monocyte chemoattracting protein 1 (MCP-1), and IL-10 were modulated as a reaction to stimulation Caco2 cells with non-pathogenic (*Lactobacillus sakei*) and enteropathogenic bacteria.

Epithelial cells indirectly communicate with other cells of the immune system; they achieve this through releasing cytokines and antimicrobial peptides (AMPs). Upon pathogenic challenge, epithelial cells secrete pro-inflammatory cytokines/chemokines (IL-8) that recruit pro-inflammatory cells from circulation such as neutrophils, or

release MCP-1 to recruit monocytes to the site of infection. In addition to cytokine secretion, they secrete AMPs, particularly human  $\beta$  defensin-2 (hBD-2) (Ganz, 2003). AMPs like hBD-2 are important regulators at gut mucosa, and result in many academics placing their emphasis on the prominent factors that regulate their expression. Tsutsumi-Ishii and Nagaoka in 2003 described a co-culture model of A549 pulmonary epithelial cells with mononuclear phagocytes (Mono-Mac-6 monocytic cells) stimulated with LPS to explore the modulation of hBD-2 in pulmonary epithelial cells (Tsutsumi-Ishii and Nagaoka, 2003). The role of LPS was found to notably up-regulated hBD-2 promoter activity, whilst A549 in itself did not react to LPS to initiate the hBD-2 promoter. IL-1 $\beta$  and TNF $\alpha$  in the culture supernatants from LPS-stimulated macrophages initiated the hBD-2 promoter in A549 cells connected with NF- $\kappa$ B instigation. This co-culture model also addressed the epithelial cell barrier role. Tanoue et al in 2008 created a co-culture system of Caco2 cells (apical side) and macrophage RAW264.7 cells (basolateral side) for scrutinising the anti-inflammatory impact of food aspects (Tanoue et al., 2008). This study might not give an accurate reflection results as using human cells in combination with a mouse cells for a co-culture system. However, in this research, the stimulation of RAW264.7 cells with LPS led to a decline in trans-epithelial electrical resistance (TEER) and was connected with an expansion in TNF $\alpha$  generation from RAW264.7 cells and IL-8 mRNA expression in Caco2 cells. Treatment with anti-TNF $\alpha$  antibodies restricted TNF $\alpha$  generation and IL-8 mRNA expression.

THP-1 cells are utilised in co-culture systems with varying cell forms, such as adipocytes, T-lymphocytes, platelets and intestinal cells. It is widely recognised that adipocytes obesity-associated inflammation improves macrophage infiltration in

adipose tissue by inflammatory cytokine generation, like TNF $\alpha$  and IL-6 (Berg and Scherer, 2005). An *in vitro* model system was established for human adipose tissue by the incubation of adipocytes (pre-adipocyte cell line) with THP-1 cells in indirect incubation with conditioned medium from THP-1 cells or else the direct co-culture of SBGS adipocytes with THP-1 cells. It was reported that macrophages contribute to regulate inflammation in human adipose tissue by secretion cytokines such as TNF $\alpha$  and CD95L that block Akt2 in near fat cells thereby inducing insulin resistance i.e. macrophages can induce apoptosis to fat cells that are insulin resistant and therefore apoptosis sensitive. (Keuper et al., 2011). A co-culture system by co-culturing predominant human adipocytes and pre-adipocytes with THP-1 cells via the use of a transwell process also established to explore the gene expression trend of M1, M2a, M2b, and M2c macrophages so they quantitated adipose macrophage phenotypes and adipose tissue fibrosis in lean and obese focuses (Spencer et al., 2010). The findings from this research suggested that the co-culture of adipocytes with either M1 or M2 macrophages resulted in a general transition of macrophage gene expression via secreting soluble elements from adipocytes, which advocate a transition of the M1 to the M2-phenotype cell during co-culture. Examples of research utilising THP-1-derived macrophages co-cultured with intestinal epithelial cells are as follows. Research conducted by Watanabe et al., 2004a indicated that the Caco2 cells barrier purposed could be disturbed by co-culturing with THP-1 macrophages distinct from PMA because of the secretion of TNF $\alpha$  by THP-1-derived macrophages (Watanabe et al., 2004a). A similar conclusion was proposed by (Moyes et al., 2010) when Caco2 cells co-cultured within a transwell process with THP-1 macrophages differentiated by PMA, generated a reduced, not so organised epithelium and enhanced micro particle uptake.

Within this chapter, an advanced transwell co-culture process was devised for the first time via a comparison of two varying status of gut physiology; inflammation (Caco-2/M1) or homeostatic (Caco-2/M2) to explore the immunomodulatory outcomes of K-12 LPS and/or TNF $\alpha$  and IL-1 $\beta$  on macrophages with or without LPS. Culturing entirely varying Caco-2 at apical compartment and THP-1 cells specified by PMA (M1-like M $\Phi$ s), or cells distinguished by VitD $_3$  (M2-like M $\Phi$ s at lower compartment, apically treated with probiotics, and basolateral stimulated with LPS was undertaken to represent gut pathology (inflammatory) and conventional homeostasis concepts, respectively. The research placed its emphasis on the role of epithelial cytokine and TEER (intestinal epithelial barrier function) recorded by cytokine production in homeostatic and chronic inflammation model.

The main questions addressed in this investigation are.

**Hypothesis 1:** co-culture model, Caco2/M1- and Caco2/M2-like M $\Phi$ s, differentially respond to *E. coli* LPS.

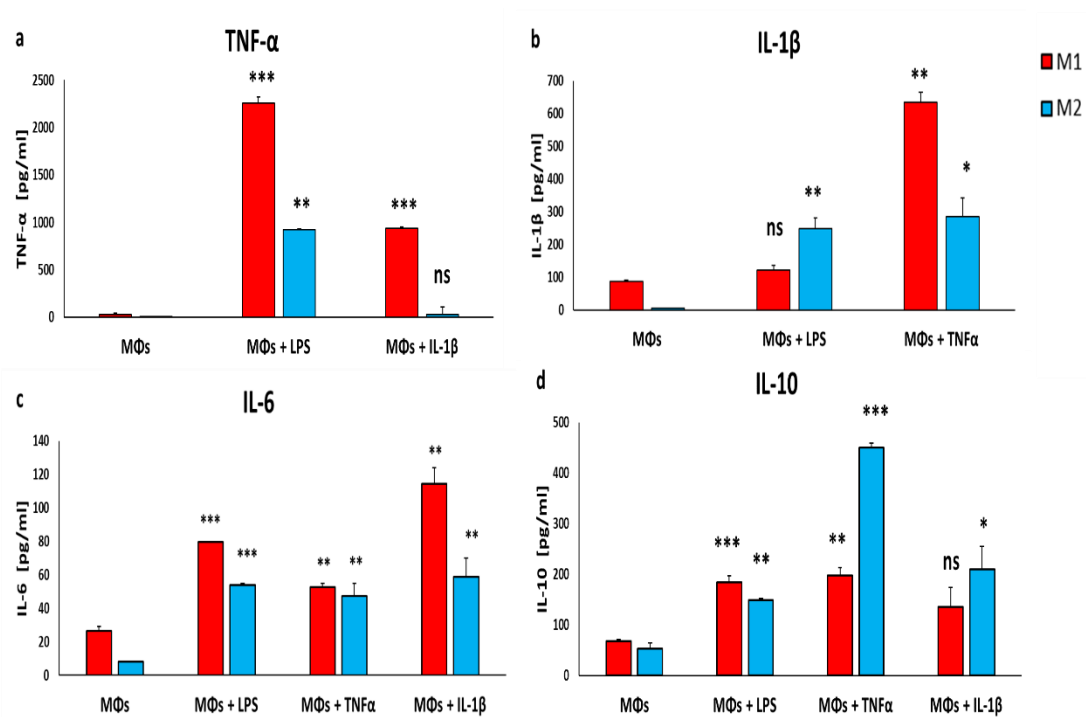
**Hypothesis 2:** pro-inflammatory cytokines, TNF $\alpha$  and IL-1 $\beta$ , can modulate K12-LPS induced cytokine production in co-culture models.

**Hypothesis 3:** Endotoxin tolerisation by *E. coli* LPS can be induced differentially by M $\Phi$  subsets in co-culture models in comparison with M $\Phi$ s monoculture.

## 5.2 Results

### 5.2.1 MΦ subsets exhibit different cytokine profiles in response to K12-LPS, TNFα and IL-1β stimulation.

In order to create a database for the models that been investigated in this chapter, behaviour of MΦ subsets in response to different stimulus were re-studied and represented differently. As described in chapter 3, MΦ subsets displayed different cytokine profiles in response upon stimulation with 100 ng/ml K12-LPS, 10 ng/ml TNFα, or 5 ng/ml IL-1β. TNFα production by IL-1β-stimulated M1-like MΦs was 50% less than TNFα produced by LPS-stimulated MΦs. However, the level of TNFα was significantly high upon stimulation by both LPS and IL-1β ( $P= 0.001$ ) compared to untreated M1-like MΦs. Interestingly, M2-like MΦs produced TNFα cytokine only in response to LPS stimulation and the level of TNFα was slightly significant high ( $P= 0.001$ ), whereas TNFα production upon IL-1β stimulation was at the low level of unstimulated cells (Figure 5.2-1 a) compare to (Figure 3.2-1). In contrast, M1-like MΦs produced IL-1β cytokine in response to LPS stimulation at very low level similar to untreated cells, whereas IL-1β level was significantly high ( $P= 0.002$ ) upon TNFα stimulation. M2-like MΦs produced a significant level of IL-1β upon stimulation by LPS ( $P= 0.008$ ) and TNFα ( $P= 0.019$ ) (Figure 5.2-1 b). Stimulation of either M1- or M2-like MΦs by LPS, TNFα or IL-1β resulted in production a significant high level of IL-6 cytokine (Figure 5.2-1 c). In contrast, both MΦ subsets secreted significant level of IL-10 cytokine upon LPS and TNFα while M2-like only produced a considerable level of IL-10 in response to IL-1β stimulated (Figure 5.2-1 d).



**Figure 5.2-1: MΦ subsets exhibit different cytokine profiles in response to K12-LPS, TNFα and IL-1β stimulation.**

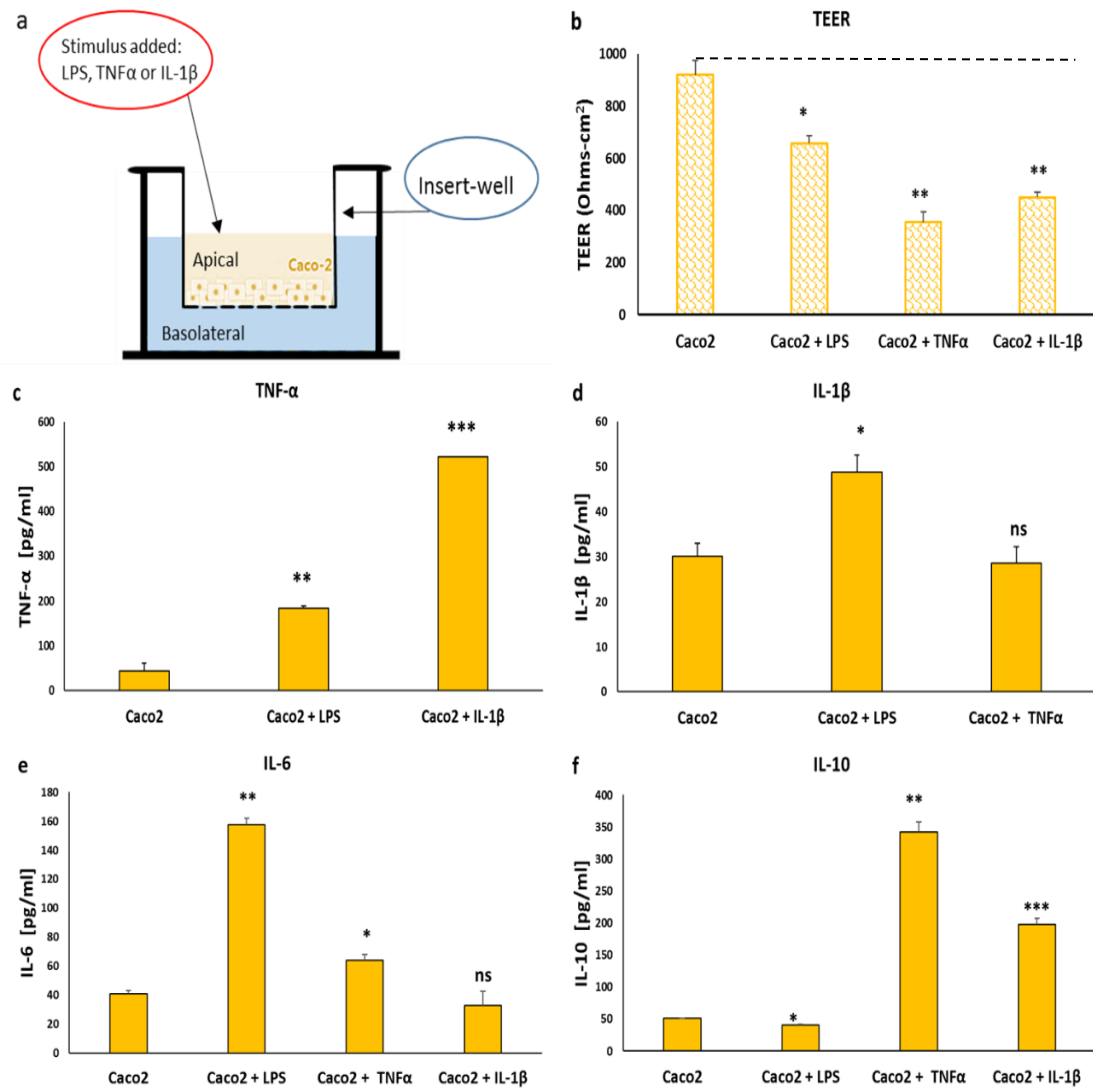
M1 (Red) and M2 (Blue) MΦ subsets were stimulated in the presence or absence of [100 ng/ml] K12-LPS, [10 ng/ml] TNFα, or [5 ng/ml] IL-1β. Cytokine production; TNFα (a), IL-1β (b), IL-6 (c) and IL-10 (d) is expressed as the mean ± SD in pg/ml. Data displayed represents duplicate samples for n= 3 replicate experiments. Significant differences in cytokine production between untreated and stimulus activated MΦs are indicated as \* p < 0.05, \*\* p < 0.01, \*\*\* P < 0.001 and ns= not significant. Note; TNFα production was not measured when stimulus is TNFα and IL-1β production was not measured when stimulus is IL-1β.

### 5.2.2 Epithelial cells exhibit cell barrier modulation and different cytokine profiles in response to K12-LPS, TNFα and IL-1β stimulation.

To create a baseline of data about Caco2 epithelial cell behaviour and epithelial barrier function in the normal homeostasis or chronic inflammation status, either LPS, TNFα or IL-1β stimulated Caco2 cells. Figure 5.2-2 showed the experiment set up diagram (a), the effect of LPS, TNFα and IL-1β stimulation on epithelial barrier function by TEER technique (b) and epithelial cytokine production profile in response these stimuli (c, d, e, f). Results showed that TEER of LPS-stimulated Caco2 dropped down by 28.6% and (P= 0.02) compared to untreated cells ( $919 \pm 55$ )  $\Omega/\text{cm}^2$ . TEER results



decreased significantly by 61% and 51% upon stimulation with TNF $\alpha$  and IL-1 $\beta$  (P= 0.003) (Figure 5.2-2 b). Caco2 epithelial cells exhibited differential cytokine profile in response to LPS, TNF $\alpha$  and IL-1 $\beta$ . LPS-stimulated Caco2 produced TNF $\alpha$  cytokine by 184 pg/ml (P= 0.007) and IL-1 $\beta$ -stimulated produced 521 pg/ml (P= 0.001) of TNF $\alpha$  cytokine (Figure 5.2-2 c). LPS-stimulated Caco2 cells secreted slightly significant higher level of IL-1 $\beta$  cytokine (P= 0.02), whereas TNF $\alpha$ -stimulated Caco2 cells secreted a very low IL-1 $\beta$  cytokine similar to unstimulated control levels (Figure 5.2-2 d). IL-6 production by Caco2 cells was significantly higher upon LPS stimulation (P= 0.002), less significant upon TNF $\alpha$  stimulation (P= 0.0160) and very low to control level upon IL-1 $\beta$  stimulation (Figure 5.2-2 e). Interestingly, Caco2 produced IL-10 cytokine in very low concentration to control level upon LPS stimulation. However, IL-10 level was significantly produced by Caco2 cells upon TNF $\alpha$  stimulation with 342 pg/ml (P= 0.002) and upon IL-1 $\beta$  stimulation 198 pg/ml (P= 0.001) (Figure 5.2-2 f).



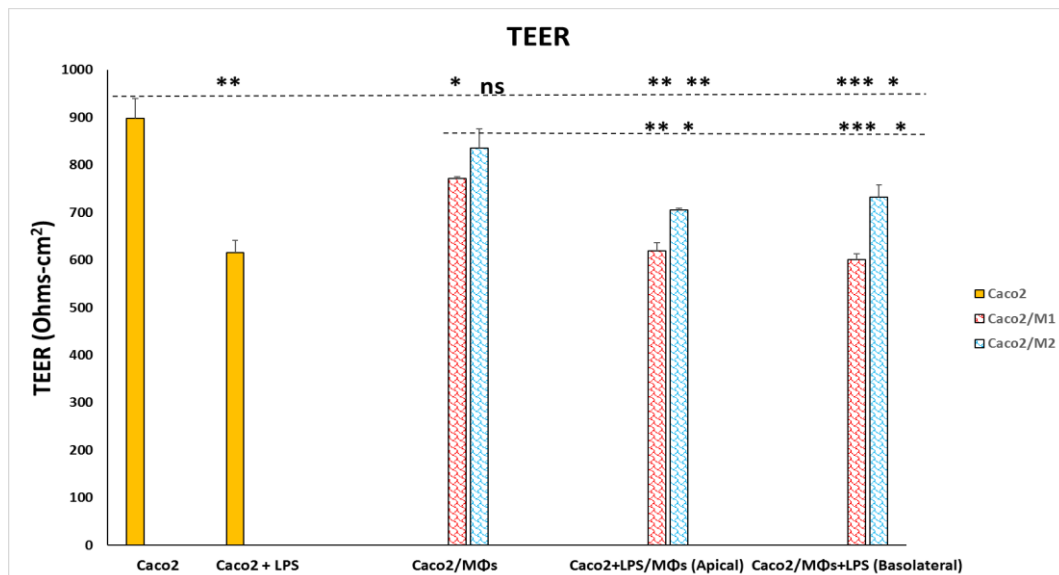
**Figure 5.2-2: Epithelial cells exhibit cell barrier modulation and different cytokine profiles in response to K12-LPS, TNF $\alpha$  and IL-1 $\beta$  stimulation (apical administration of inflammatory stimuli).**

Caco-2 epithelial cells were differentiated in transwell inserts for 21 days followed by incubation in the presence or absence of [100 ng/ml] K12-LPS, [10 ng/ml] TNF $\alpha$ , or [5 ng/ml] IL-1 $\beta$  for 24h (a). TEER measurements (b) were performed using EVOM epithelial voltmeter for Caco-2 epithelial cells. Cytokine production is expressed as the mean  $\pm$  SE in pg/ml for TNF- $\alpha$  (c), IL-1 $\beta$  (d), IL-6 (e) and IL-10 (f) secreted into apical compartment. Data displayed represents duplicate samples for n= 3 replicate experiments. Significant differences in cytokine production between stimulus-activated Caco-2 epithelial cells and unstimulated Caco2 cells are indicated as \* p < 0.05, \*\* p < 0.01, \*\*\* P < 0.001 and ns= not significant. Note; TNF $\alpha$  production was not measured when stimulus is TNF $\alpha$  and IL-1 $\beta$  production was not measured when stimulus is IL-1 $\beta$ .

### **5.2.3 Barrier modulation and cytokine production induced by K12-LPS in co-culture models; caco-2/M1 and caco-2/M2.**

In order to evaluate endotoxin role in modulation of the co-culture models, caco-2/M1 and caco-2/M2, and epithelial barrier function in normal homeostasis or chronic inflammation environment, K12-LPS was added to the co-culture either to apical side to mimic a homeostasis status or to basolateral side to mimic an inflammation status.

Firstly, Figure 5.2-3 showed TEER measurements in single Caco2 cells and both co-culture models in the presence or absence of LPS. Results displayed that TEER of untreated single Caco2 cells ( $898 \pm 42$ )  $\Omega/\text{cm}^2$  decreased significantly by 31% to ( $615 \pm 27$ )  $\Omega/\text{cm}^2$  ( $P= 0.002$ ) upon LPS stimulation. TEER dropped down after culturing with M $\Phi$  subsets, however, TEER value of Caco2/M1 was significantly lower than TEER of untreated cells ( $P= 0.02$ ) , whereas it the reduction was not significant by Caco2/M2 ( $P= 0.171$ ). TEER values of co-culture of Caco2/M1-like M $\Phi$ s decreased significantly in the presence of LPS in apical side by 31% ( $P= 0.002$ ) and basolateral side by 33% ( $P= 0.0008$ ) comparing to untreated cells. They also decreased significantly in apical side by 19% ( $P= 0.001$ ) and basolateral side by 23% ( $P= 0.00075$ ) comparing to untreated Caco2/M1-like M $\Phi$ s. However, comparing to untreated cells, the reduction of TEER values of co-culture of Caco2/M2-like M $\Phi$ s was slightly significant in the presence of LPS in apical side by 21% ( $P= 0.005$ ) and basolateral side by 18% ( $P= 0.014$ ). The reduction of TEER values of Caco2/M2-like M $\Phi$ s, comparing to untreated Caco2/M2-like M $\Phi$ s, was slightly significant LPS in apical side by 15% ( $P= 0.015$ ) and basolateral side by 13% ( $P= 0.047$ ) (Figure 5.2-3).



**Figure 5.2-3: Epithelial cells barrier modulation induced by K12-LPS in Caco2 and co-culture models, caco2/M1-like MΦs and caco2/M2-like MΦs.**

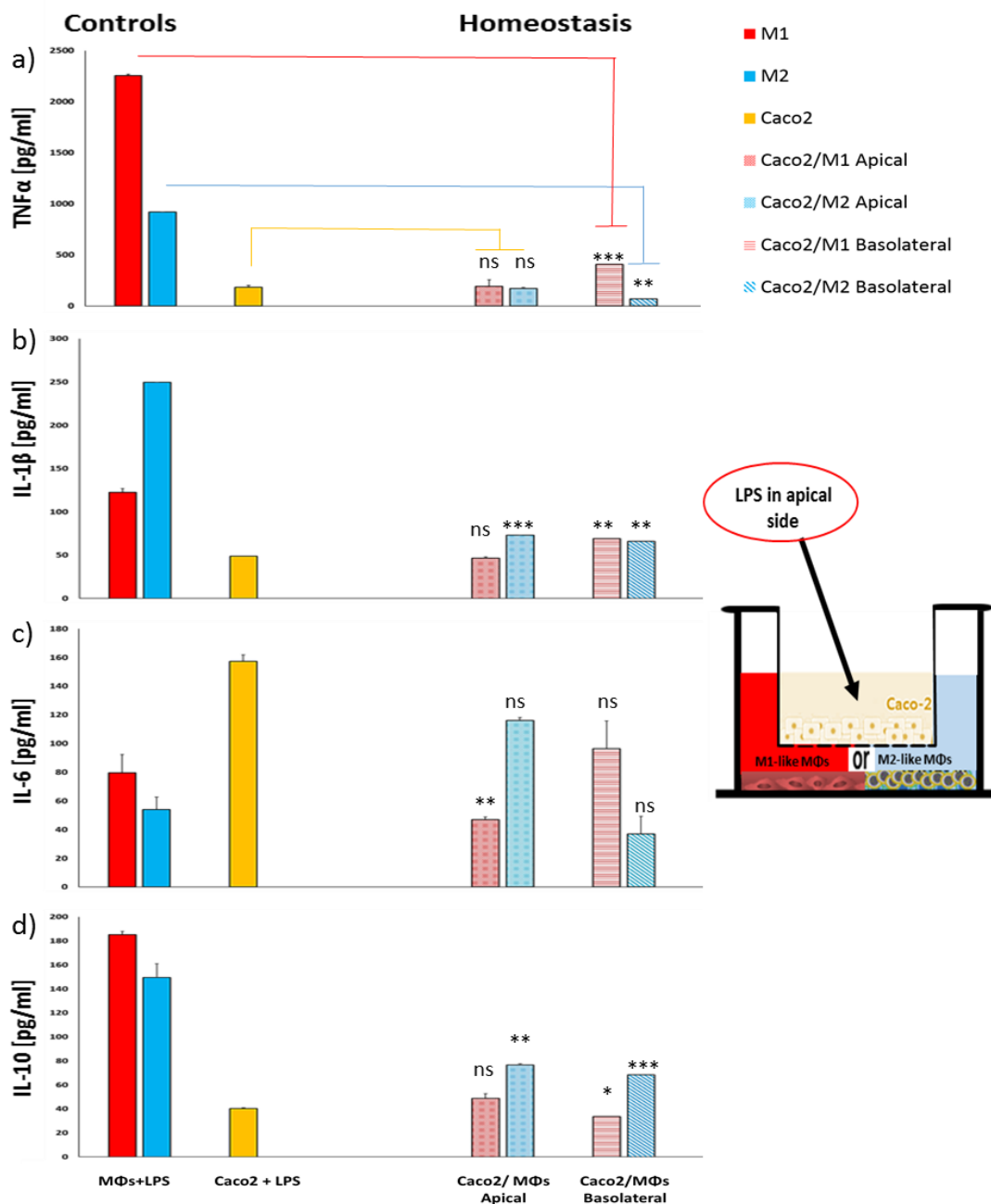
Caco-2 epithelial cells were differentiated in transwell inserts for 21 days. Three groups are shown include single Caco2 (yellow), co-culture of Caco2/M1- (net red) or Caco2/M2-like (net blue) MΦs where LPS was added either to Caco2 (apical) or to MΦ subsets (basolateral). K12-LPS concentration was 100 ng/ml and the incubation period was 18h. TEER measurements were performed using an EVOM epithelial voltmeter. Data displayed is a representative experiment with duplicate samples of n= 3 replicate experiments. Significant effects compared to the control are indicated as \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 and ns= not significant.

Secondly, Pro- and anti-inflammatory cytokine production by co-cultured Caco2/M1-like and Caco2/M2-like MΦs was analysed where K12-LPS was added to apical side (homeostasis status) or to basolateral side (inflammation status). Secreted cytokine was measured by two sides in co-culture model i.e. cytokine production by Caco2 from apical supernatant and cytokine production by MΦ subsets either M1-like or M2-like from basolateral supernatant. So, results in figures below are identified as (1) mono-culture (M1-like, M2-like MΦ and Caco2), (2) apical supernatant and basolateral supernatant for Caco2/M1-like MΦ and (3) apical supernatant and basolateral supernatant for Caco2/M2-like MΦ.

Homeostasis status in this results mean when LPS is added to apical side i.e. to Caco2 cells in co-culture models (Figure 5.2-4). TNF $\alpha$  cytokine production in apical supernatant by both Caco2/M1-like and Caco2/M2-like M $\Phi$ s showed similar level to TNF $\alpha$  produced by single Caco2 upon LPS stimulation. However, upon apical LPS stimulation, TNF $\alpha$  cytokine level in basolateral supernatant significantly reduced by Caco2/M1-like P= 0.001 and Caco2/M2-like M $\Phi$ s P= 0.004 compared to monoculture M1-like and M2-like M $\Phi$ s (Figure 5.2-4 a). Apical supernatant of Caco2/M1-like displayed similar level of IL-1 $\beta$  compared to single Caco2 upon LPS stimulation, whereas apical supernatant of Caco2/M2-like exhibited significant increase in IL-1 $\beta$  P= 0.001. IL-1 $\beta$  production in basolateral supernatant significantly decreased by Caco2/M1-like P= 0.002 and Caco2/M2-like M $\Phi$ s P= 0.01 upon apical LPS stimulation compared to monoculture M1-like and M2-like M $\Phi$ s (Figure 5.2-4 b). Caco2/M1-like M $\Phi$ s showed a significant reduction in IL-6 production in apical supernatant P= 0.002, whereas there was no significant change in all other supernatants (Figure 5.2-5 c). In apical supernatant, Caco2/M1-like M $\Phi$ s displayed similar level of IL-10 compared to single Caco2 upon apical LPS stimulation while Caco2/M2-like M $\Phi$ s, in apical supernatant, exhibited significant increase in IL-10 P= 0.01. However, IL-10 production in basolateral supernatant significantly decreased by Caco2/M1-like M $\Phi$ s P= 0.031 and Caco2/M2-like M $\Phi$ s P= 0.001 upon apical LPS stimulation compared to monoculture M1-like and M2-like M $\Phi$ s (Figure 5.2-4 d).

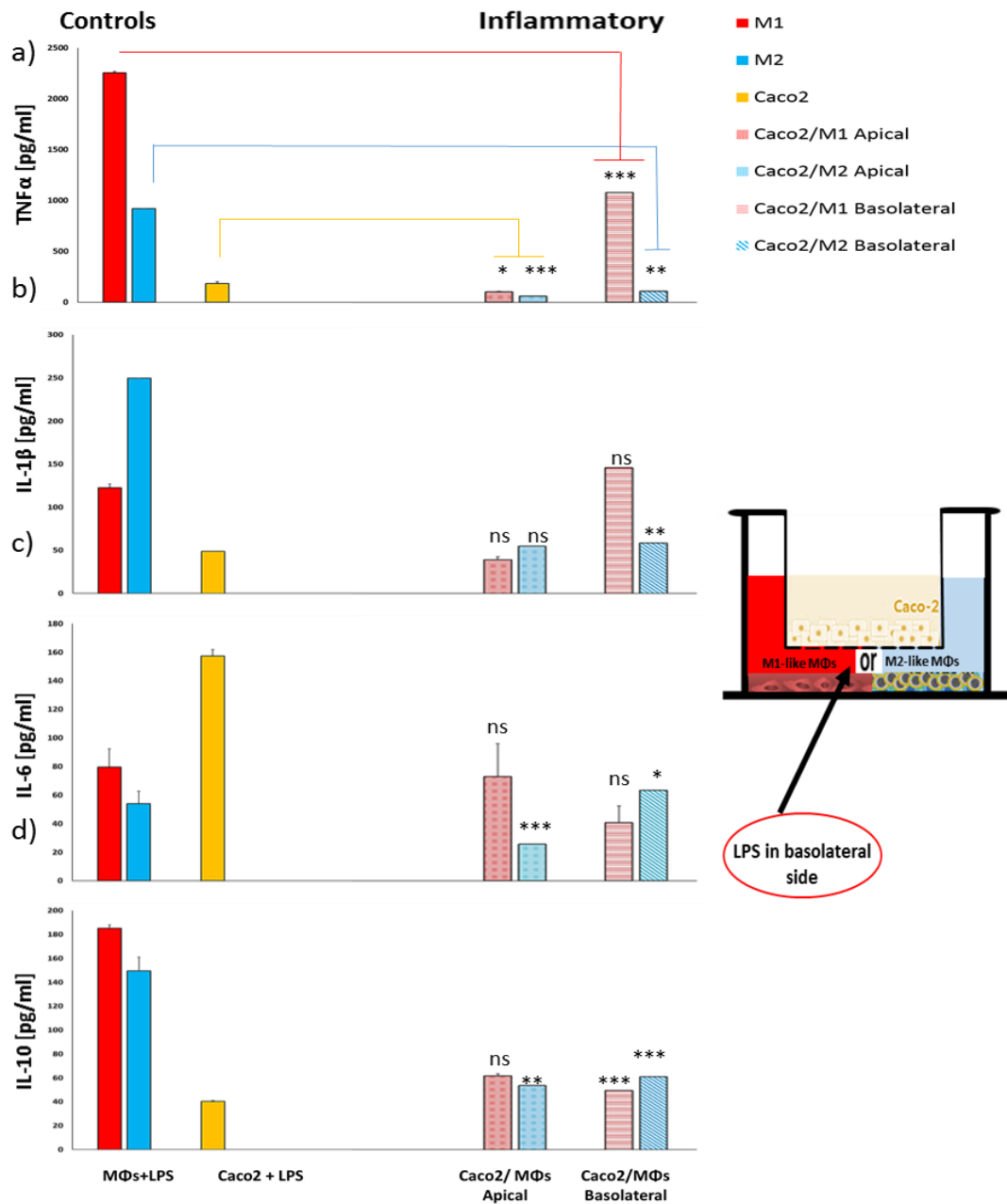
In inflammatory environment where LPS was added to basolateral side of the epithelial cell barrier, i.e. to M $\Phi$  cells underneath in co-culture models, the results were slight different (Figure 5.2-5). Compared to monoculture (M1-like, M2-like M $\Phi$  and Caco2), TNF $\alpha$  production was significantly decreased upon LPS treatment by both Caco2/M1-

like and Caco2/M2-like MΦ apical and basolateral side. (P= 0.012 for Caco2/M1-like apical side, P= 0.0001 Caco2/M1-like basolateral side, P= 0.001 for Caco2/M2-like apical side and P= 0.004 Caco2/M2-like basolateral side) (Figure 5.2-5 a). Upon basolateral LPS stimulation, Caco2/M2-like MΦs showed a significant reduction in IL-1β production in basolateral supernatant P= 0.01, whereas there was no significant change in all other supernatants (Figure 5.2-5 b). Caco2/M1-like MΦs showed no significant change in IL-6 production in both apical and basolateral supernatant. However, in apical side of Caco2/M2-like MΦs IL-6 was significantly reduced P= 0.001, while, interestingly, IL-6 slightly significant increased by Caco2/M2-like MΦs in basolateral side P= 0.001 upon basolateral LPS stimulation (Figure 5.2-5 c). In apical supernatant, Caco2/M1-like MΦs presented similar level of IL-10 compared to single Caco2 upon basolateral LPS stimulation while Caco2/M2-like MΦs, in apical supernatant, exhibited significant increase in IL-10 P= 0.006. However, IL-10 production in basolateral supernatant significantly decreased by Caco2/M1-like MΦs P= 0.001 and Caco2/M2-like MΦs P< 0.001 upon basolateral LPS stimulation compared to monoculture M1-like and M2-like MΦs (Figure 5.2-5 d).



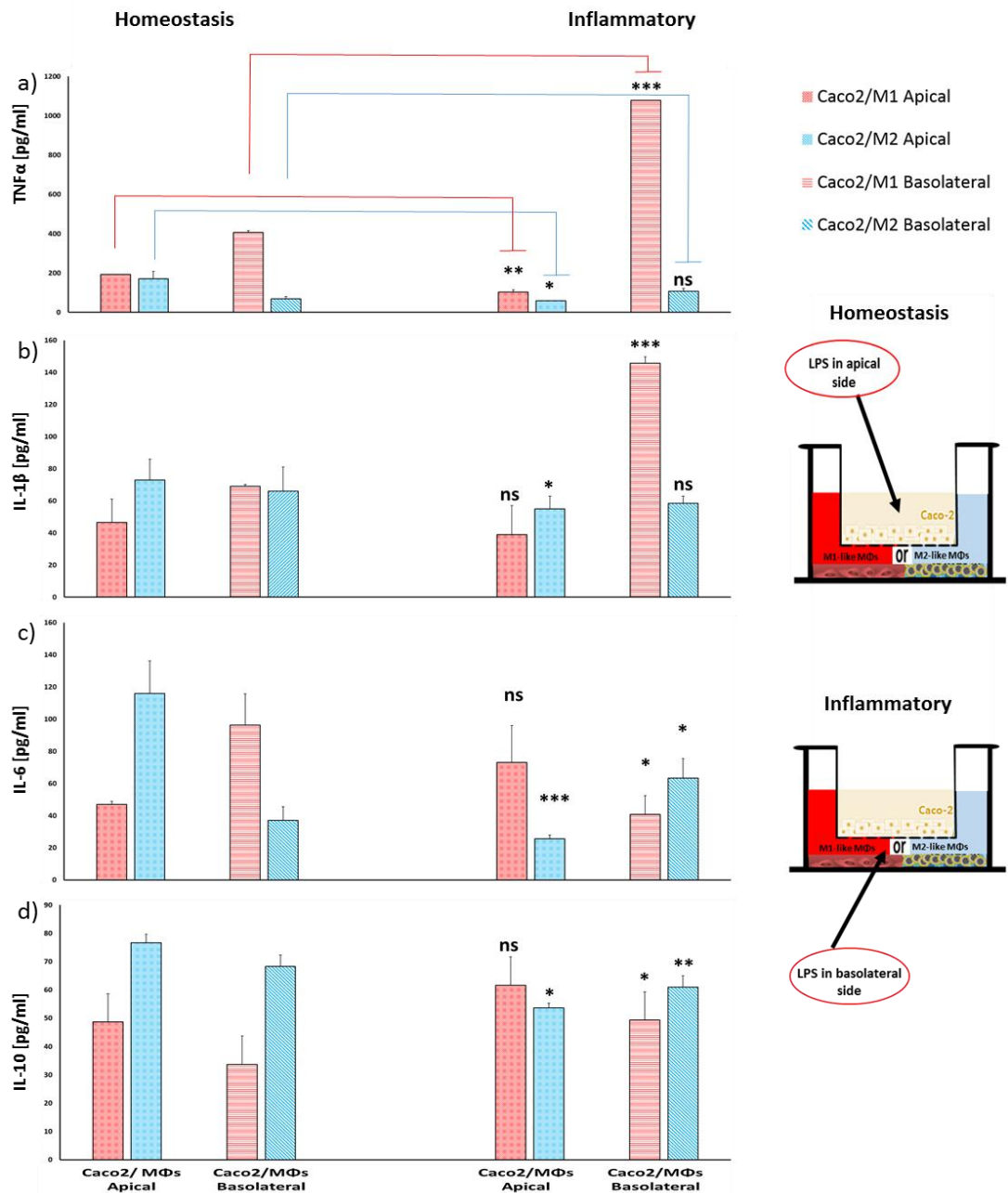
**Figure 5.2-4: Cytokine production by co-culture models, Caco2/MΦ subsets, in homeostatic environment.**

Single cultures of M1-, M2-like MΦs, Caco2 cells and co-culture models of Caco2/M1 and Caco2/M2 cells were stimulated with 100 ng/ml of K12-LPS and the incubation period was 18h. LPS was added to the apical side in the co-culture models. M1 (red), M2, (blue), Caco2 (yellow), Caco2/M1 apical supernatant (net red), Caco2/M2 apical supernatant (net blue), Caco2/M1 basolateral supernatant (strip red) and Caco2/M2 basolateral supernatant (strip red). Cytokine production is expressed as the mean  $\pm$  SD in pg/ml for TNFα (a), IL-1β (b), IL-6 (c) and IL-10 (d). Data displayed represents duplicate samples for n= 2 replicate experiments. Significant differences in cytokine production between K12-LPS activated M1-, M2-like MΦs or Caco2 and the co-culture models are indicated as \* p < 0.05, \*\* p < 0.01, \*\*\* P < 0.001 and ns= not significant.





The comparison in the previous results was between positive control results and the results of homeostasis or inflammatory treatments. However, these results can be presented differently. It might be a good observation when compare the results of this co-culture model in homeostasis environment to the same model in an inflammatory environment. The production of the pro-inflammatory cytokine,  $\text{TNF}\alpha$ , in the apical supernatant of Caco2/ MΦs significantly decreased in inflammatory environment in both co-culture models  $P= 0.009$  for Caco2/M1-like and  $P= 0.048$  for Caco2/M2-like MΦs. Interestingly,  $\text{TNF}\alpha$  significantly increased  $P= 0.001$  in Caco2/M1-like MΦs basolateral supernatant in inflammatory environment compared to homeostasis environment, while there was no significant change in Caco2/M2-like MΦs basolateral supernatant (Figure 5.2-6 a). There was no significant change in  $\text{IL-1}\beta$  level in the apical supernatant of Caco2/M1, whereas it slightly decreased in Caco2/M2 supernatant  $P= 0.015$ . However, remarkably,  $\text{IL-1}\beta$  significantly increased in basolateral Caco2/M1 supernatant  $P< 0.001$ , whereas as no significant change in the basolateral supernatant of Caco2/M2 (Figure 5.2-6 b). There was no significant change in  $\text{IL-6}$  level in the apical supernatant of Caco2/M1, whereas it decreased in Caco2/M2 supernatant  $P= 0.001$ . Conversely,  $\text{IL-6}$  significantly decreased in basolateral Caco2/M1 supernatant  $P= 0.02$ , whereas it significantly increased in the basolateral supernatant of Caco2/M2  $P= 0.039$  (Figure 5.2-6 c). The production of the anti-inflammatory cytokine,  $\text{IL-10}$ , in the apical supernatant of Caco2/M1-like MΦs in inflammatory environment was similar to  $\text{IL-10}$  level in homeostasis environment. On the other hand,  $\text{IL-10}$  slightly decreased in the apical supernatant of Caco2/M2-like MΦs ( $P= 0.029$ ). Interestingly, in Caco2/M1-like MΦs,  $\text{IL-10}$  significantly increased ( $P= 0.015$ ), while in Caco2/M2-like MΦs,  $\text{IL-10}$  significantly decreased ( $P= 0.006$ ) in inflammatory environment compared to homeostasis environment (Figure 5.2-6 d).

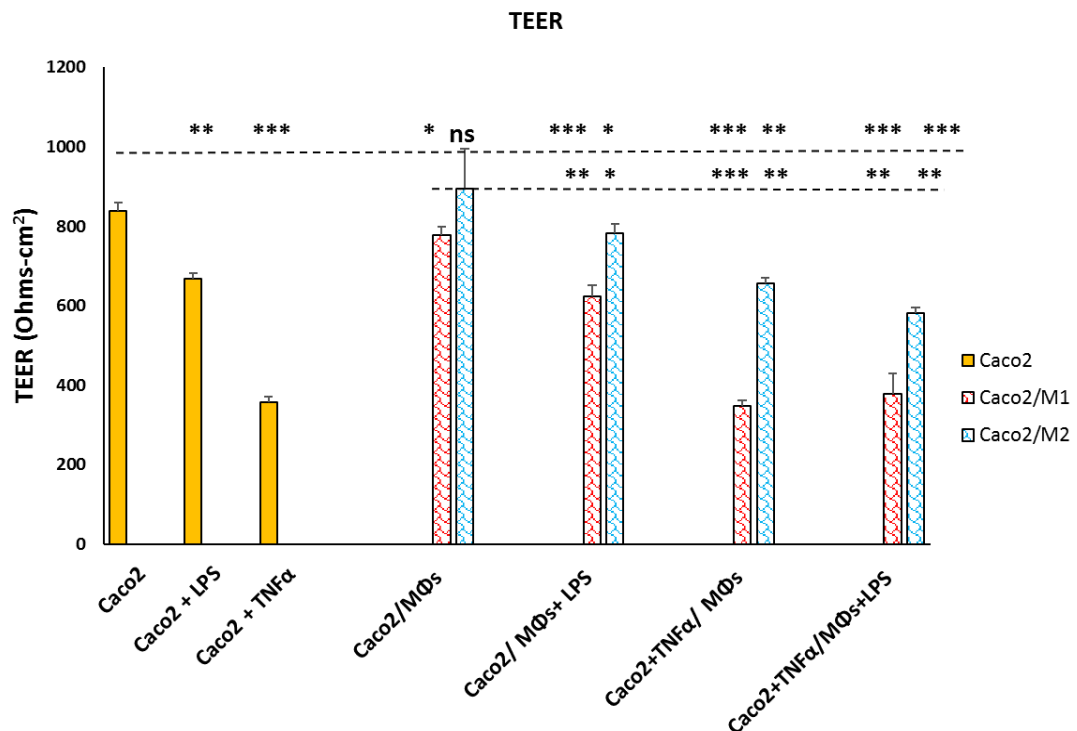


**Figure 5.2-6: Cytokine production by co-culture models, Caco2/MΦ subsets, in homeostasis and inflammatory environment.**

Co-culture models of Caco2/M1 and Caco2/M2 cells were stimulated with 100 ng/ml of K12-LPS and the incubation period was 18h. LPS was added to the apical side (homeostasis) or basolateral side (inflammatory) in the co-culture models. Caco2/M1 apical supernatant (net red), Caco2/M2 apical supernatant (net blue), Caco2/M1 basolateral supernatant (strip red) and Caco2/M2 basolateral supernatant (strip red). Cytokine production is expressed as the mean  $\pm$  SD in pg/ml for TNFα (a), IL-1β (b), IL-6 (c) and IL-10 (d). Data displayed represents duplicate samples for  $n = 2$  replicate experiments. Significant differences in cytokine production between homeostasis state and inflammatory state are indicated as \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $P < 0.001$  and ns= not significant. Note, this figure is a re-representation of figures Figure 5.2-4 and Figure 5.2-5 directly comparing homeostasis with inflammatory

#### **5.2.4 TNF $\alpha$ modulation of barrier function and cytokines production in different co-culture models.**

The first aim was to evaluate epithelial cell barrier function, TEER measurement was used to determine the epithelial cell integrity. Caco2 cells ( $838 \pm 20$ )  $\Omega/\text{cm}^2$  decreased significantly by 57% to ( $356 \pm 15$ )  $\Omega/\text{cm}^2$  ( $P= 0.001$ ) upon TNF $\alpha$  stimulation. In Caco2/M1 co-culture model comparing to untreated Caco2/M1, TEER was significantly reduced by all stimuli treatments. To illustrate, TEER value decreased significantly by 20% ( $P= 0.0001$ ) upon LPS stimulation, 55% ( $P= 0.0006$ ) upon stimulation by TNF $\alpha$  alone and 53% ( $P= 0.002$ ) upon TNF $\alpha$  stimulation followed by LPS. However, data showed that reduction in TEER by Caco2/M2 co-culture model was less significant than suppression in Caco2/M1 co-culture model. TEER value, comparing to untreated Caco2/M2, decreased by 13% ( $P= 0.047$ ) upon LPS stimulation, 28% ( $P= 0.007$ ) upon stimulation by TNF $\alpha$  alone and 36% ( $P= 0.002$ ) upon stimulation by TNF $\alpha$  followed by LPS (Figure 5.2-7).

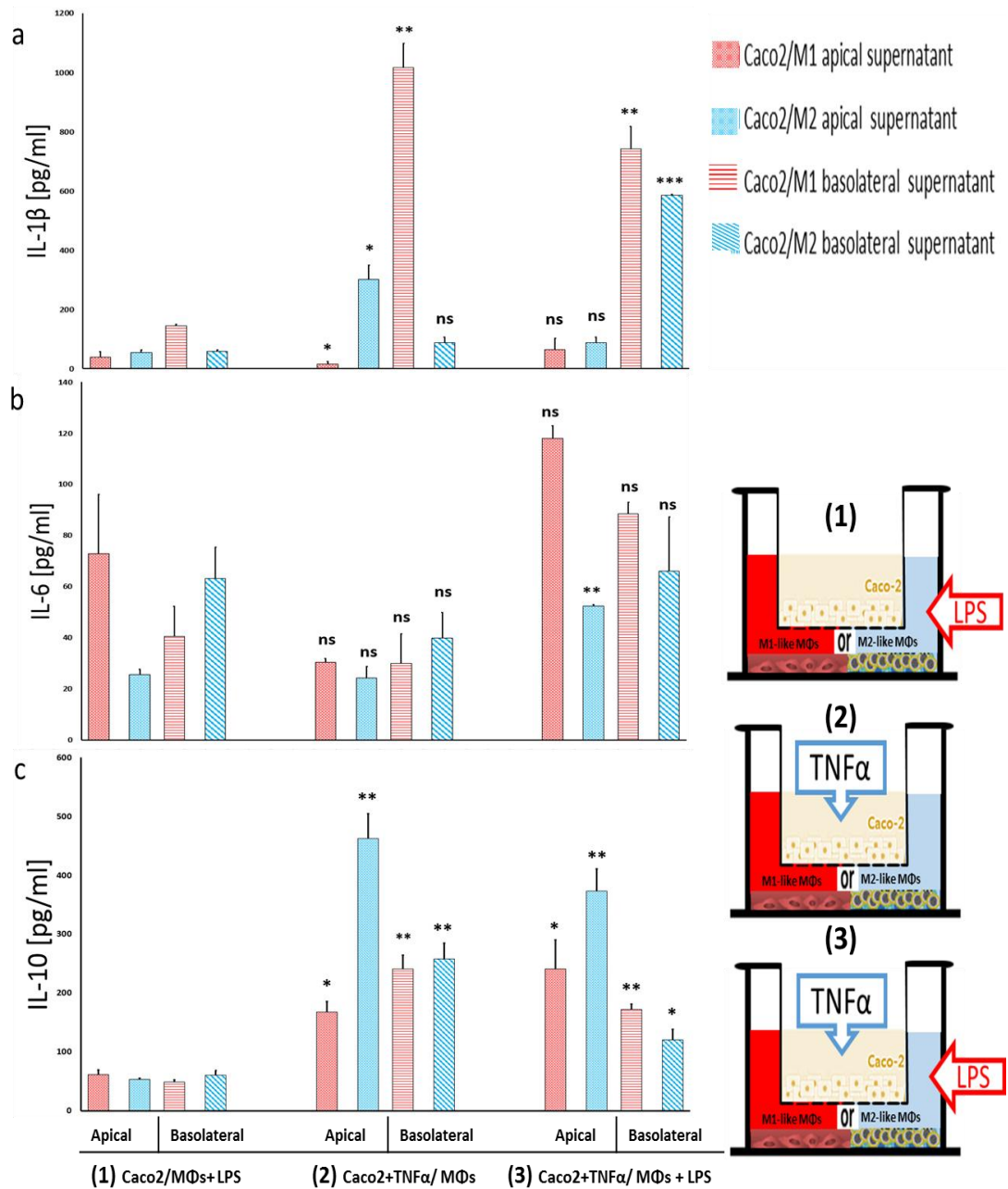


**Figure 5.2-7: TNFα modulated LPS-simulated epithelial cell barrier in Caco2 and co-culture models.**

Single Caco2 (yellow), co-culture of Caco2/M1- (net red) or Caco2/M2-like MΦs (net blue). Single Caco2 cells were stimulated by either 100 ng/ml LPS for 18h or 10 ng/ml TNFα for 24h. Co-culture models were stimulated by 100 ng/ml LPS for 18h in basolateral side, 10 ng/ml TNFα for 24h in apical side or 10 ng/ml TNFα for 24h in apical side prior to treatment with 100 ng/ml LPS for 18h in basolateral side. TEER measurements were performed using an EVOM epithelial voltmeter. Data displayed is a representative experiment with duplicate samples of n= 3 replicate experiments. Significant effects compared to the control are indicated as \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 and ns= not significant.

The second aim was to analyse cell/cell interactions between Caco2 cells and MΦ subsets, i.e. MΦ subsets effects on epithelial cell (homeostasis) and epithelial cells effects on MΦ subsets (inflammation) by measuring the cytokine level upon these different treatments. TNFα cytokine measurement was excluded to avoid any confusion as TNFα was used as a stimulus. Therefore, the measured cytokines were IL-

1 $\beta$ , IL-6 and IL-10 and the comparison was applied between homeostasis (LPS treatment to basolateral surface) and inflammatory environment (TNF $\alpha$  with and without LPS). Upon apical treatment with TNF $\alpha$  only, IL-1 $\beta$  in Caco2/M2 apical supernatant (P= 0.018) and Caco2/M1 basolateral supernatant (P= 0.004) was significantly higher than IL-1 $\beta$  in same supernatants in homeostasis environment. There was no significant change in IL-1 $\beta$  level in the apical supernatant of both Caco2/M1- and Caco2/M2-like M $\Phi$ s upon TNF $\alpha$  challenge prior to LPS basolateral treatment. However, IL-1 $\beta$  level significantly increased in the basolateral supernatant of both Caco2/M1- (P= 0.008) and Caco2/M2-like M $\Phi$ s (P< 0.001) (Figure 5.2-8 a). Interestingly, there was no significant difference in IL-6 level upon TNF $\alpha$ -stimulation or TNF $\alpha$  challenge prior LPS stimulation compared to LPS stimulation. The only change was in the apical supernatant of Caco2/M2-like M $\Phi$ s upon TNF $\alpha$  challenge prior to LPS basolateral stimulation, which showed a significant increase in IL-6 (P= 0.003) (Figure 5.2-8 b). Surprisingly, in all supernatant, IL-10 production was extremely high upon TNF $\alpha$  stimulation and TNF $\alpha$  challenge followed by LPS re-challenge in basolateral side. Upon treatment with TNF $\alpha$  alone, the p value of the apical supernatant of Caco2/M1- was (P= 0.018) and Caco2/M2-like M $\Phi$ s (P= 0.005) and for basolateral supernatant of Caco2/M1- (P= 0.007) and Caco2/M2-like M $\Phi$ s (P= 0.009). Upon treatment with TNF $\alpha$  prior to LPS challenge, the p value of the apical supernatant of Caco2/M1- was (P= 0.036) and Caco2/M2-like M $\Phi$ s (P= 0.007) and for basolateral supernatant of Caco2/M1- (P= 0.003) and Caco2/M2-like M $\Phi$ s (P= 0.034) (Figure 5.2-8 d).

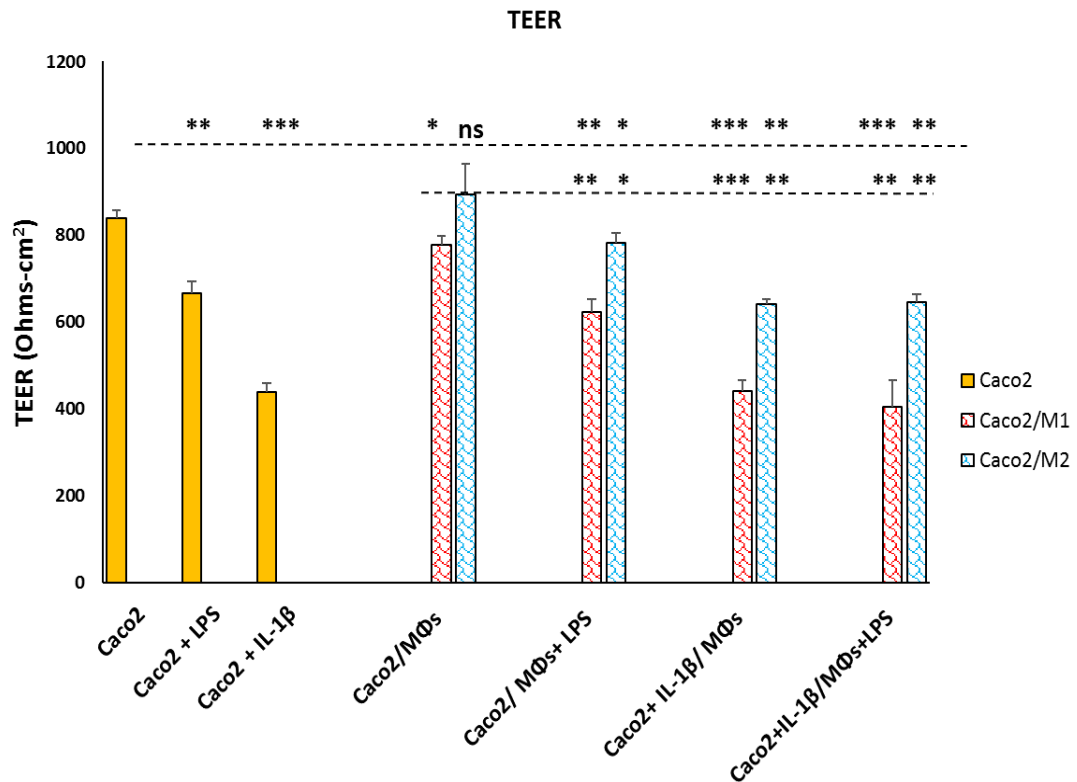


**Figure 5.2-8: Differential sensitivity of LPS-induced co-culture model cytokines to TNFα.**

Co-culture models were stimulated by: (1) 100 ng/ml LPS in basolateral side for 18h, (2) 10 ng/ml TNFα in apical side for 24h or (3) 10 ng/ml TNFα in apical side for 24h prior to treatment with 100 ng/ml LPS in basolateral side for 18h. Caco2/M1 apical supernatant (net red), Caco2/M2 apical supernatant (net blue), Caco2/M1 basolateral supernatant (striped red) and Caco2/M2 basolateral supernatant (striped blue). Cytokine production is expressed as the mean  $\pm$  SD in pg/ml for IL-1β (a), IL-6 (b) and IL-10 (c). Data displayed represents duplicate samples for n = 2 replicate experiments. Significant differences in cytokine production between TNFα modulated LPS-induced cells are indicated as \* p < 0.05, \*\* p < 0.01, \*\*\* P < 0.001 and ns = not significant.

### **5.2.5 IL-1 $\beta$ modulation of barrier function and cytokine production and in different M $\Phi$ subsets/co-culture models.**

Firstly, to determine the epithelial cell integrity, TEER measurement was applied. Caco2 cells ( $838 \pm 20$ )  $\Omega/\text{cm}^2$  decreased significantly by 47% to ( $439 \pm 20$ )  $\Omega/\text{cm}^2$  ( $P=0.001$ ) upon IL-1 $\beta$  stimulation. In Caco2/M1 co-culture model, TEER was significantly suppressed by all stimuli treatments. TEER value decreased significantly comparing to untreated Caco2/M1 by 44% ( $P=0.0001$ ) upon stimulation by IL-1 $\beta$  alone and 48% ( $P=0.002$ ) upon IL-1 $\beta$  stimulation followed by LPS. However, similar to data in (Figure 5.2-7), data showed that disruption of barrier (reduction in TEER) by Caco2/M2 co-culture model was less significant than suppression in Caco2/M1 co-culture model. TEER value decreased by 28% upon stimulation by either IL-1 $\beta$  alone and ( $P=0.005$ ) or IL-1 $\beta$  followed by LPS and ( $P=0.006$ ) (Figure 5.2-9).



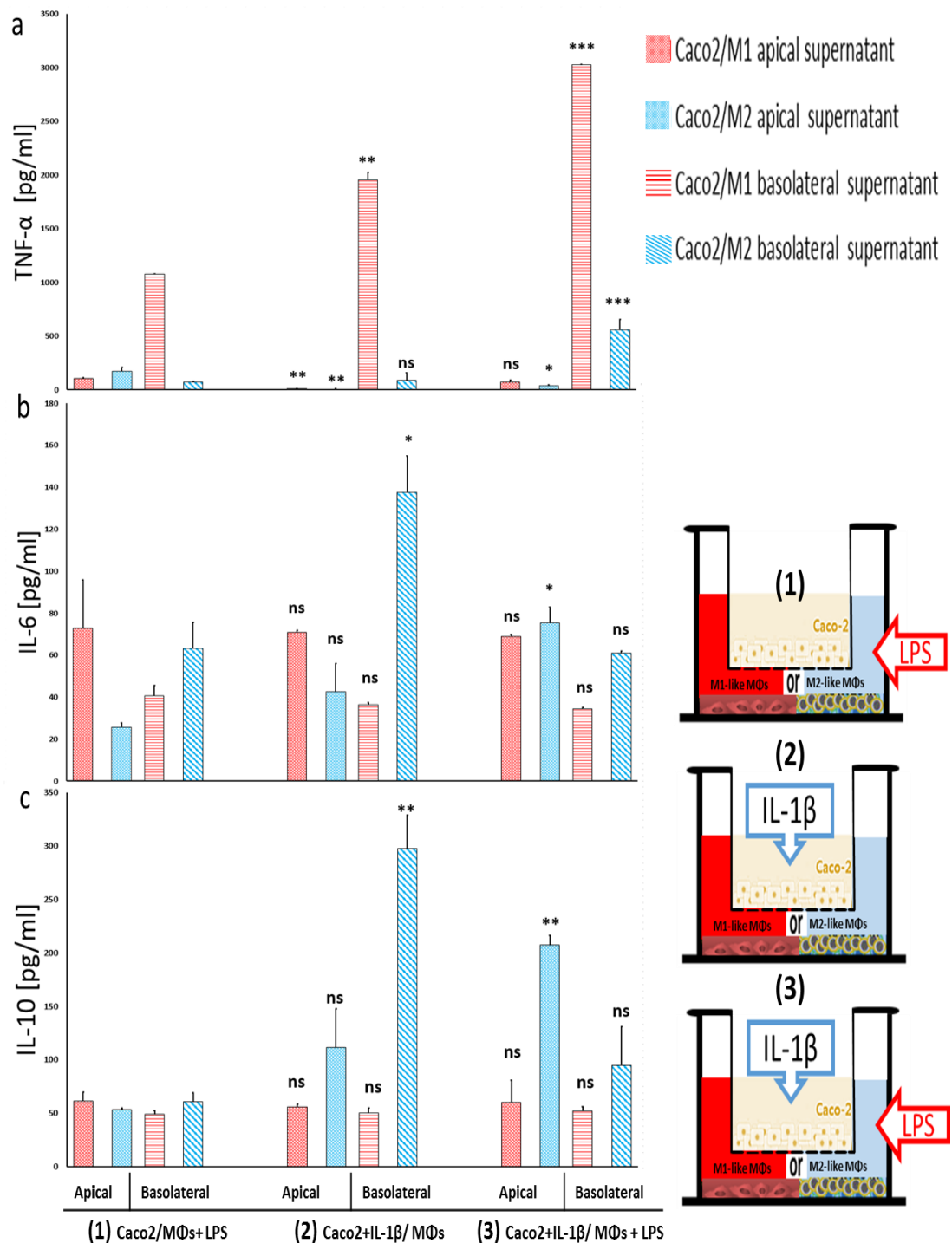
**Figure 5.2-9: Modulation of LPS-induced epithelial cells barrier to IL-1β.**

Single Caco2 (yellow), co-culture of Caco2/M1- (net red) or Caco2/M2-like MΦs (net blue). 100 ng/ml LPS for 18h or 5 ng/ml IL-1β for 24h stimulated single Caco2 cells. Co-culture models were stimulated by 100 ng/ml LPS for 18h in basolateral side, 5 ng/ml IL-1β for 24h in apical side or 5 ng/ml IL-1β for 24h in apical side prior to treatment with 100 ng/ml LPS for 18h in basolateral side. TEER measurements were performed using an EVOM epithelial voltmeter. Data displayed is a representative experiment with duplicate samples of n= 3 replicate experiments. Significant effects compared to the control are indicated as \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 and ns= not significant.

Secondly, upon apical treatment with IL-1β alone compared to LPS stimulation, TNFα production was significant low in the apical supernatant of both Caco2/M1- (P= 0.010)



and Caco2/M2-like MΦs ( $P= 0.006$ ), whereas TNF $\alpha$  level was significantly higher in the basolateral supernatant of Caco2/M1-like MΦs ( $P= 0.003$ ) whereas the basolateral supernatant of Caco2/M2-like MΦs showed no significant change. Moreover, upon apical treatment with IL-1 $\beta$  prior to basolateral LPS stimulation, the apical supernatant of Caco2/M1 showed no significant change, while apical supernatant of Caco2/M2 showed slight decrease in TNF $\alpha$  ( $P= 0.027$ ). Interestingly, TNF $\alpha$  production was significantly higher in the basolateral supernatant of both Caco2/M1- ( $P= 0.001$ ) and Caco2/M2-like MΦs ( $P= 0.001$ ) (Figure 5.2-10 a). There was no significant change in IL-6 level in the apical supernatant of Caco2/M1 and Caco2/M2 and the basolateral supernatant of Caco2/M1-like MΦs upon apical treatment with IL-1 $\beta$  alone, whereas, interestingly, it was significantly higher in the basolateral supernatant of Caco2/M2-like MΦs ( $P= 0.037$ ). Likewise, upon apical treatment with IL-1 $\beta$  prior to basolateral LPS stimulation, there was no significant change in IL-6 level in the apical supernatant of Caco2/M1 and the basolateral supernatant of Caco2/M1- and Caco2/M2-like MΦs, while IL-6 was significantly higher in the apical supernatant of Caco2/M2-like MΦs ( $P= 0.012$ ) (Figure 5.2-10 b). Similar to IL-6, there was no significant change in IL-10 level in the apical supernatant of Caco2/M1 and Caco2/M2 and the basolateral supernatant of Caco2/M1-like MΦs upon apical treatment with IL-1 $\beta$  alone, whereas, interestingly, IL-10 level was significantly higher in the basolateral supernatant of Caco2/M2-like MΦs ( $P= 0.008$ ). Also, upon apical treatment with IL-1 $\beta$  prior to basolateral LPS stimulation, there was no significant change in IL-10 level in the apical supernatant of Caco2/M1 and the basolateral supernatant of Caco2/M1- and Caco2/M2-like MΦs, while IL-10 was significant high in the apical supernatant of Caco2/M2-like MΦs ( $P= 0.002$ ) (Figure 5.2-10 c).



**Figure 5.2-10: IL-1 $\beta$  differentially modulated LPS-induced co-culture models cytokines.**

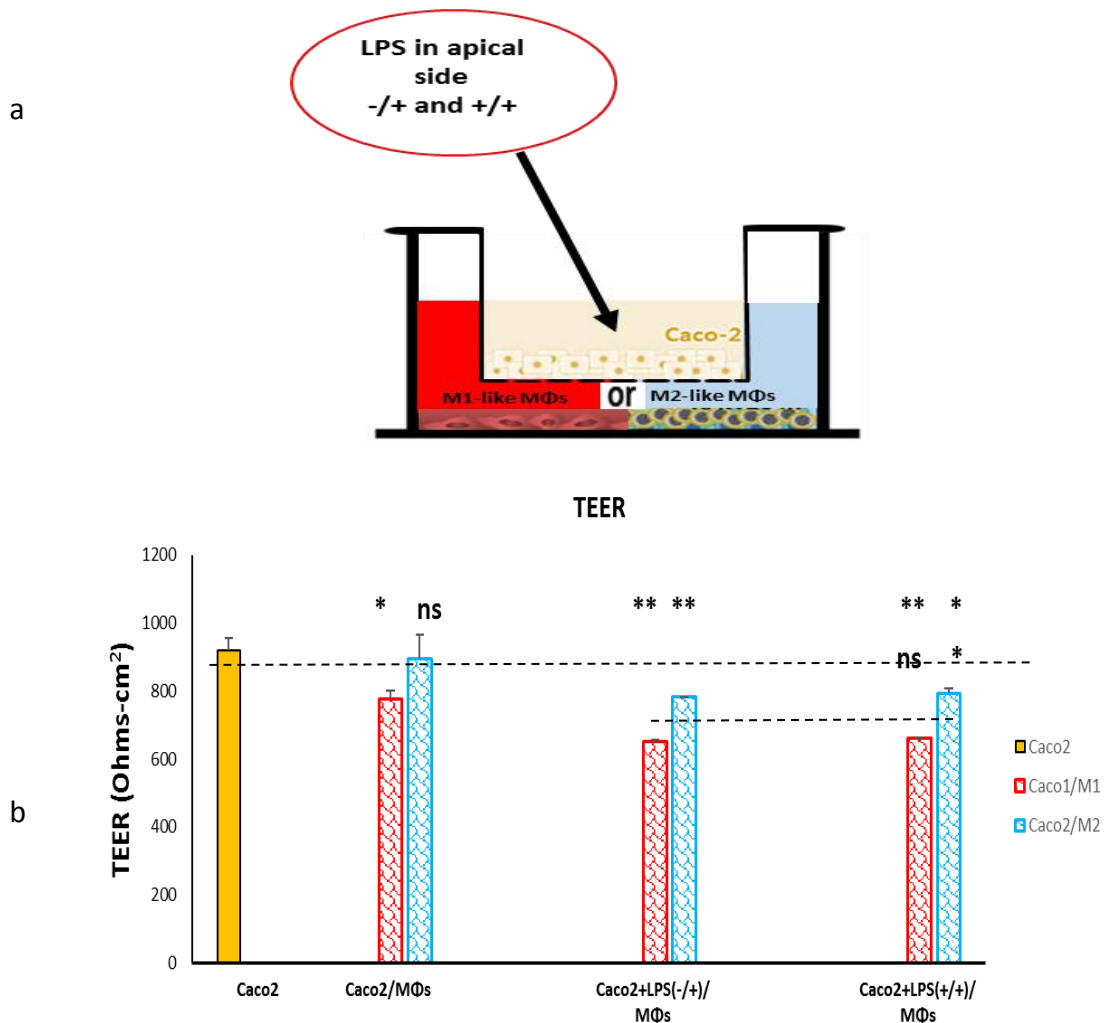
Co-culture models were stimulated by: (1) 100 ng/ml LPS in basolateral side for 18h, (2) 5 ng/ml IL-1 $\beta$  in apical side for 24h or (3) 5 ng/ml IL-1 $\beta$  in apical side for 24h prior to treatment with 100 ng/ml LPS in basolateral side for 18h. Caco2/M1 apical supernatant (net red), Caco2/M2 apical supernatant (net blue), Caco2/M1 basolateral supernatant (striped red) and Caco2/M2 basolateral supernatant (striped blue). Cytokine production is expressed as the mean  $\pm$  SD in pg/ml for TNF $\alpha$  (a), IL-6 (b) and IL-10 (c). Data displayed

represents duplicate samples for n= 2 replicate experiments. Significant differences in cytokine production between IL-1 $\beta$  modulated LPS-induced cells are indicated as \* p < 0.05, \*\* p < 0.01, \*\*\* P < 0.001 and ns= not significant.

### **5.2.6 Endotoxin tolerisation and barrier modulation induced by K12-LPS in epithelial cells/M $\Phi$ subsets co-culture models.**

In order to expand more investigation about ET, effect of ET on co-culture of epithelial cells and M $\Phi$ s in co-culture model was investigated. The main target in this investigation is M $\Phi$ s subsets, however, as Caco2 cells are involved in this model, ET effect on Caco2 was considered. ET research on co-culture model, Caco2/M1 and Caco2 M2 M $\Phi$ s, was applied in two different ways; (1) treatment of LPS as pre-stimulus and stimulus was added to apical side (Caco2 cells), (2) treatment of LPS as pre-stimulus and stimulus was added to basolateral cells (either M1- or M2-like M $\Phi$ s). The first readout was identifying the direct and indirect effect of ET on epithelial barrier function by measuring TEER. Interestingly, the trend of results of TEER upon ET on apical side (direct) and basolateral side (indirect) was similar (Figure 5.2-11 and Figure 5.2-12). Results of direct effect of ET in (Figure 5.2-11) showed that compared to TEER of untreated Caco2 cells ( $918 \pm 35$ )  $\Omega/\text{cm}^2$ , TEER significantly decreased by 30% (P= 0.002) in both stimulated and tolerised Caco2/M1 M $\Phi$ s, while TEER decreased by 15% (P= 0.005) in stimulated Caco2/M2 M $\Phi$ s similarly 15% but (P= 0.02) in tolerised cells. Similarly, TEER results of indirect effect of ET significantly decreased by 32% (P= 0.001) in stimulated Caco2/M1 M $\Phi$ s and 25% (P= 0.04) in tolerised Caco2/M1 M $\Phi$ s, while TEER slightly decreased by 15% (P= 0.014) in stimulated Caco2/M2 M $\Phi$ s similarly 15% but (P= 0.02) in tolerised cells (Figure 5.2-12). However, the more important readout is to show the significant effects of the comparison between the control (-/+ ) and tolerisation treatment (+/+). Interestingly, there was no significant change in TEER

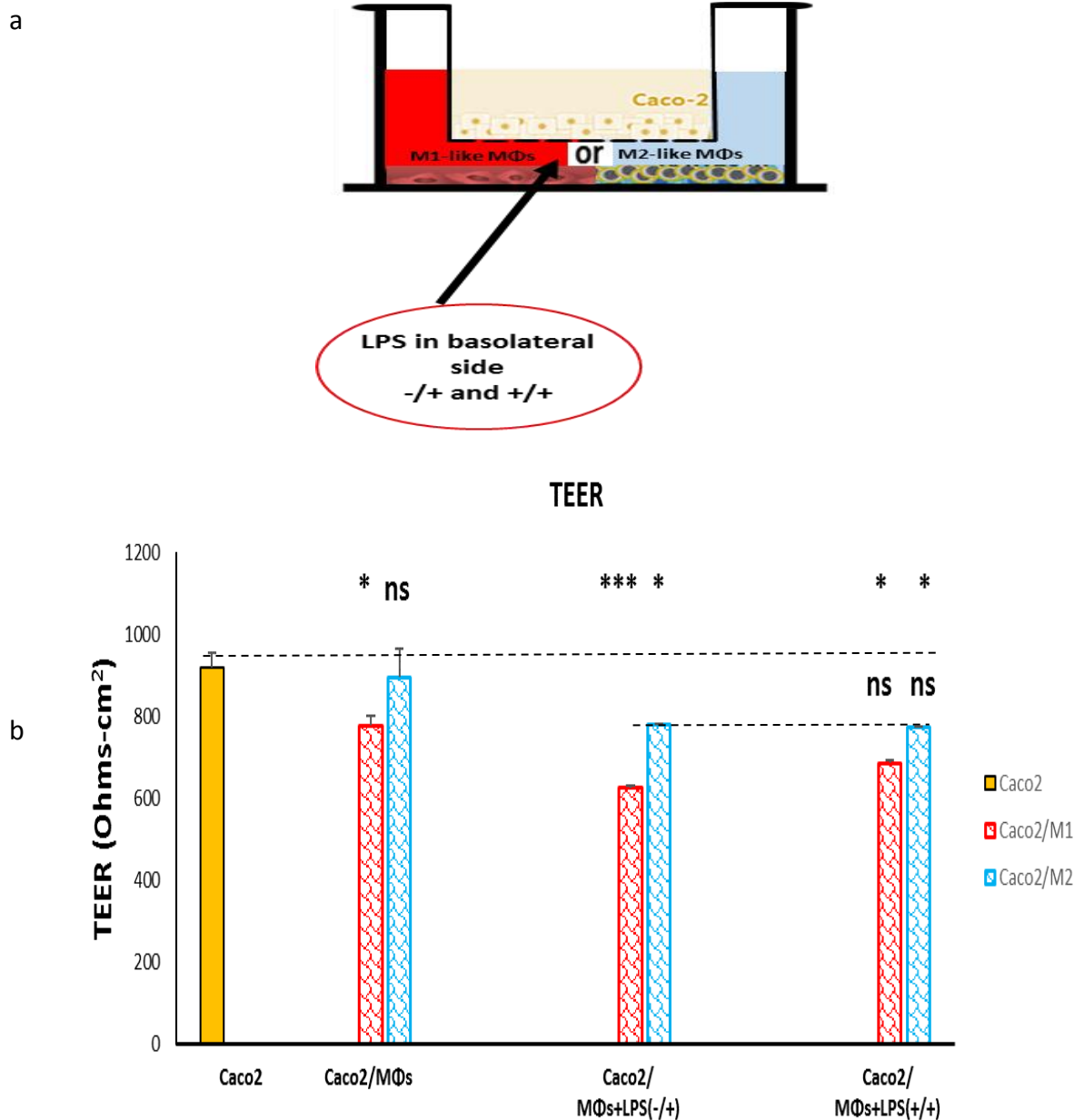
level ( $P=0.135$ ) of Caco2/M1 MΦs (+/+) upon ET on apical side (direct) compared to control (-/+), while TEER increased by 10% ( $P=0.033$ ) in tolerised Caco2/M2 MΦs (+/+) compared to stimulated Caco2/M2 MΦs (-/+) (Figure 5.2-11). All the same, there was no significant change in TEER level in both Caco2/M1 MΦs (+/+)  $P=0.114$  and Caco2/M2 MΦs (+/+)  $P=0.353$  upon ET on basolateral side (indirect) compared to stimulated Caco2/MΦs (-/+) (Figure 5.2-12).



**Figure 5.2-11: Direct effect of endotoxin tolerisation on TEER in co-culture models (Apical LPS stimulation).**

Caco-2 epithelial cells were differentiated in transwell inserts for 21 days (a). Single Caco2 (yellow), co-culture of Caco2/M1-like MΦs (net red) or Caco2/M2-like MΦs (net blue) in the presence or absence of LPS, where LPS was added to Caco2 (apical). Caco2 were pre-stimulated with 100 ng/ml K12-LPS for 24h prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS, whereas (+) = LPS. TEER measurements were performed using an EVOM epithelial voltmeter. Data displayed is a representative experiment with duplicate samples of  $n=2$  replicate experiments.

Significant effects compared between the control (-/+) and tolerisation treatment (+/+) are indicated as \*  $P < 0.05$ , \*\*  $P < 0.01$  and ns= not significant. Diagram shows how LPS was added (b).



**Figure 5.2-12: Indirect effect of endotoxin tolerisation on TEER in co-culture models (Basolateral LPS stimulation).**

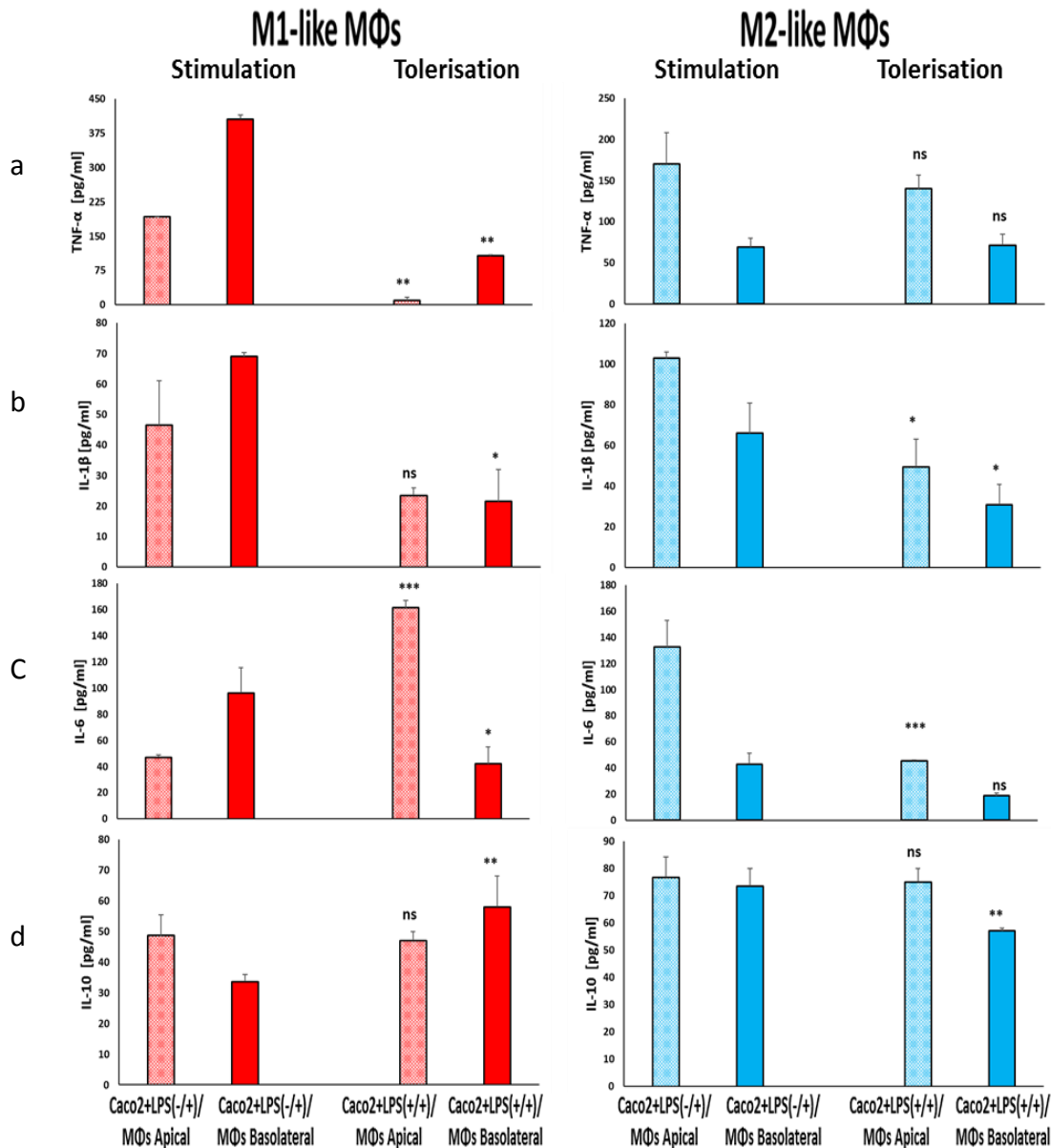
Caco-2 epithelial cells were differentiated in transwell inserts for 21 days (a). Single Caco2 (yellow), co-culture of Caco2/M1-like MΦs (net red) or Caco2/M2-like MΦs (net blue) in the presence or absence of LPS, where LPS was added to Caco2 (basolateral). M1- and M2-like MΦs were pre-stimulated with 100 ng/ml K12-LPS for 24h prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18h (-) = no LPS, whereas (+) = LPS. TEER measurements were performed using an EVOM epithelial voltmeter. Data displayed is a representative experiment with duplicate samples of  $n = 2$  replicate experiments. Significant effects compared between the control (-/+) and tolerisation treatment (+/+) are indicated as \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  and ns= not

significant. Diagram shows how LPS was added (b).

The second readout was finding the modulation of ET, whether added to apical or basolateral side, on pro- and anti-inflammatory cytokine production by both epithelial cells and macrophage subsets in co-culture models.

Firstly, data in (Figure 5.2-13) showed the effect of ET on cytokine production where LPS was added to apical side of co-culture model i.e. to Caco2 cells. In co-culture model (Caco2/M1-like MΦs), TNFα cytokine was significantly suppressed upon LPS tolerisation by Caco2 cells by 95% ( $P= 0.002$ ) and by M1-like MΦs by 74% ( $P= 0.01$ ). However, there was no significant change in TNFα production in Caco2/M2-like MΦs by either Caco2 cells or M2-like MΦs (Figure 5.2-13 a). Apical supernatant of Caco2 cells in (Caco2/M1-like MΦs) exhibited no significant change in IL-1β cytokine upon ET, whereas basolateral supernatant, M1-like MΦs, in the same model significantly suppressed IL-1β by 70% ( $P= 0.024$ ). In co-culture model (Caco2/M2-like MΦs), IL-1β level was suppressed by Caco2 cells by 50% ( $P= 0.03$ ) and by M2-like MΦs by 50% ( $P= 0.096$ ) (Figure 5.2-13 b). Remarkably, IL-6 cytokine was significantly up-regulated by 70% ( $P= 0.001$ ) by Caco2 cells, while it was slightly down-regulated by 50% ( $P= 0.049$ ) by M1-like MΦs in (Caco2/M1-like MΦs) upon endotoxin tolerisation. On the other hand, in (Caco2/M2-like MΦs), IL-6 was significantly down-regulated by 65% ( $P= 0.001$ ) by Caco2 cells, whereas M2-like MΦs showed no significant change upon endotoxin tolerisation (Figure 5.2-13 c). Caco2 cells in both co-culture model, (Caco2/M1-like MΦs and Caco2/M2-like MΦs), exhibited no significant change in IL-10 level upon LPS tolerisation compared to LPS stimulation. Nevertheless, in basolateral side in co-culture, IL-10 cytokine level was significantly up-regulated by M1-like MΦs by 42% ( $P=$

0.005), whereas, interestingly, it was significantly down-regulated by M2-like MΦs by 23% (P= 0.006) (Figure 5.2-13 d).

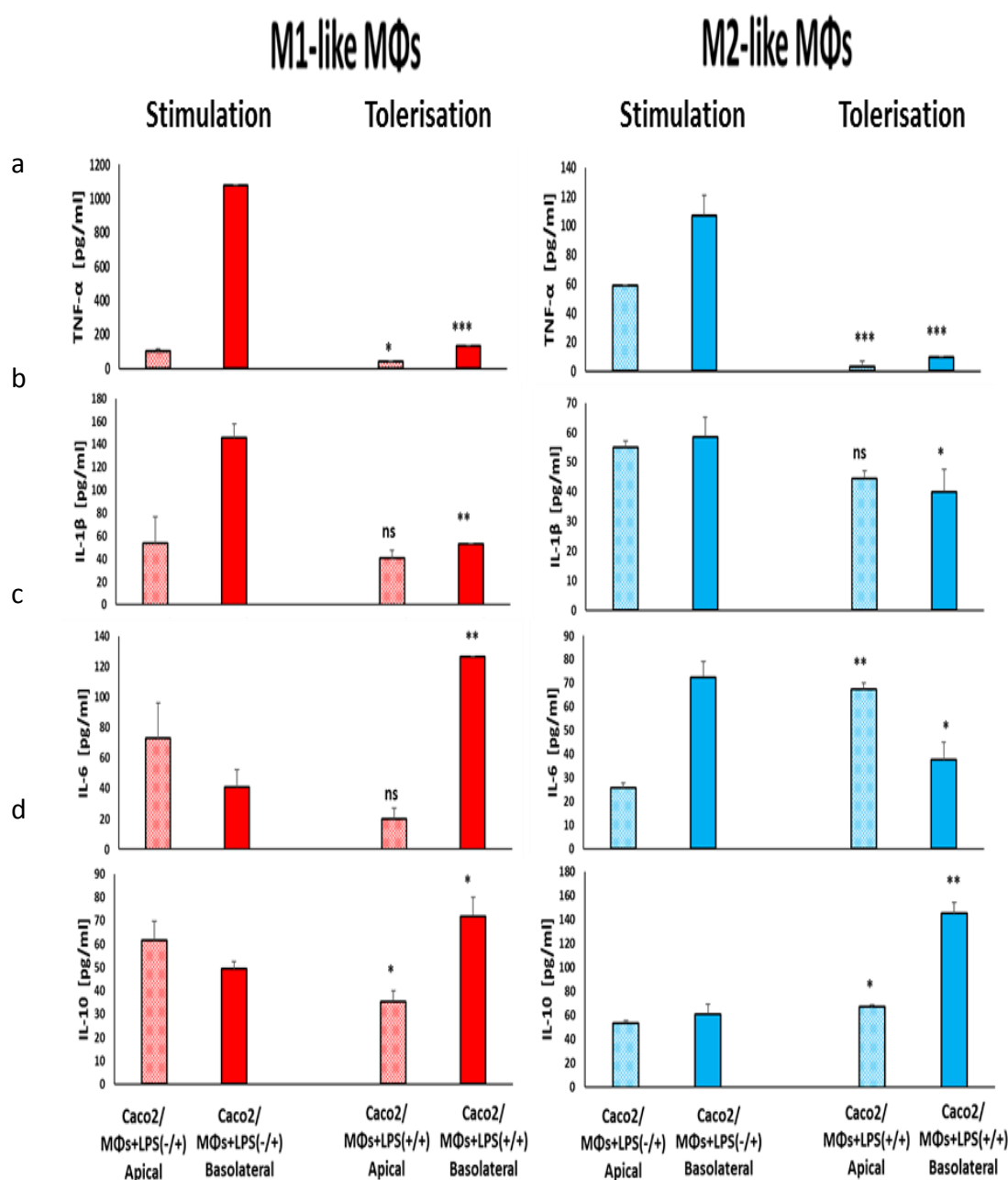


**Figure 5.2-13: Effects of endotoxin tolerisation on co-culture model in homeostatic state (Apical LPS stimulation).**

Caco2 cells in co-culture of Caco2/M1-like MΦs or Caco2/M2-like MΦs were incubated with or without 100 ng/ml LPS for 24h prior to stimulation with 100 ng/ml LPS for a further 18h as (-) = no LPS, whereas (+) = LPS i.e. LPS added in apical side. Caco2/M1 apical supernatant (net red), Caco2/M1 basolateral supernatant (red), Caco2/M2 apical supernatant (net blue), and Caco2/M2 basolateral supernatant (blue). Cytokine production is expressed as the mean  $\pm$  SD in pg/ml for TNF $\alpha$  (a), IL-1 $\beta$  (b), IL-6 (c) and IL-10 (d). Data displayed represents duplicate samples for n= 2 replicate experiments. Significant differences in cytokine production between untolerised (-/+) and tolerised (+/+) MΦs are indicated as \* p < 0.05, \*\* p < 0.01, \*\*\* P < 0.001 and ns= not significant.

Secondly, data in (Figure 5.2-14) presented the influence of ET on cytokine production where LPS was added to basolateral side i.e. representative of barrier disruption and chronic inflammation to MΦ subsets in co-culture model. In both co-culture model (Caco2/M1-like MΦs and Caco2/M2-like MΦs), TNFα cytokine was significantly suppressed upon LPS tolerisation either apical or basolateral supernatant. In Caco2/M1-like MΦs, TNFα suppression by Caco2 by 60% (P= 0.20) and M1-like MΦs by 88% (P=0.001). Likewise, in Caco2/M2-like MΦs, TNFα suppression by Caco2 by 93% (P=0.001) and M1-like MΦs by 91% (P= 0.001) (Figure 5.2-14 a). Apical supernatant of Caco2 cells in (Caco2/M1-like MΦs) presented no significant change in IL-1β cytokine upon ET, whereas M1-like MΦs in the same model significantly suppressed IL-1β by 64% (P= 0.009). Furthermore, Caco2/M2-like MΦs co-culture showed same sort of response to ET as Caco2 cells displayed no significant change in IL-1β level, while M2-like MΦs significantly suppressed IL-1β by 33% (P= 0.028)(Figure 5.2-14 b). There was no significant change in IL-6 level induced by Caco2 cells, while, surprisingly, IL-6 cytokine was significantly up-regulated by 43% (P= 0.004) by M1-like MΦs in (Caco2/M1-like MΦs) upon endotoxin tolerisation. In contrast, in (Caco2/M2-like MΦs), IL-6 was significantly up-regulated by 62% (P= 0.003) by Caco2 cells and significantly down-regulated by 48% (P= 0.036) by M2-like MΦs upon endotoxin tolerisation (Figure 5.2-14 c). Caco2 cells in (Caco2/M1-like MΦs) exhibited significant reduction in IL-10 cytokine by 42% (P= 0.05) upon ET, whereas M1-like MΦs in the same model significantly increased IL-10 cytokine by 32% (P= 0.047). IL-10 cytokine was significantly up-regulated by 20% (P= 0.013) by Caco2 cells and 58% (P= 0.005) by M2-like MΦs upon endotoxin tolerisation (Figure 5.2-14 d).





**Figure 5.2-14: Effects of endotoxin tolerisation on co-culture model in inflammatory state (Basolateral LPS stimulation).**

MΦs in co-culture of Caco2/M1-like MΦs or Caco2/M2-like MΦs were incubated with or without 100 ng/ml LPS for 24h prior to stimulation with 100 ng/ml LPS for a further 18h as (-) = no LPS, whereas (+) = LPS i.e. LPS added in basolateral side. Caco2/M1 apical supernatant (net red), Caco2/M1 basolateral supernatant (red), Caco2/M2 apical supernatant (net blue), and Caco2/M2 basolateral supernatant (blue). Cytokine production is expressed as the mean  $\pm$  SD in pg/ml for TNF $\alpha$  (a), IL-1 $\beta$  (b), IL-6 (c) and IL-10 (d). Data displayed represents duplicate samples for n= 2 replicate experiments. Significant differences in cytokine production between untolerised and tolerised MΦs are indicated as \* p < 0.05, \*\* p < 0.01, \*\*\* P < 0.001 and ns= not significant.

### 5.2.7 Summary of chapter 5 results

**Table 5.2-1: Epithelial barrier sensitivity (TEER measurements).**

	Epithelial barrier	TEER	
		measurement $\Omega/\text{cm}^2$	%
	Caco2	898	100
	Caco2 /M1	771	86 ↓
	Caco2 /M2	836	93 ↓
	Caco2 + LPS	615	68 ↓
<b>homeostatic</b>	Caco2 + LPS /M1	619	69 ↓
	Caco2 + LPS /M2	705	79 ↓
<b>inflammatory</b>	Caco2/M1 + LPS	601	67 ↓
	Caco2/M1 + LPS	732	82 ↓
<b>TNF<math>\alpha</math> stimulation</b>	Caco2+ TNF $\alpha$	356	40 ↓
	Caco2+ TNF $\alpha$ / M1	348	39 ↓
	Caco2+ TNF $\alpha$ / M2	656	73 ↓
	Caco2+ TNF $\alpha$ / M1 + LPS	377	42 ↓
	Caco2+ TNF $\alpha$ / M2 + LPS	580	65 ↓
<b>IL-1<math>\beta</math> Stimulation</b>	Caco2+ IL-1 $\beta$	439	49 ↓
	Caco2+ IL-1 $\beta$ / M1	440	49 ↓
	Caco2+ IL-1 $\beta$ / M2	641	71 ↓
	Caco2+ IL-1 $\beta$ / M1 + LPS	406	45 ↓
	Caco2+ IL-1 $\beta$ / M2 + LPS	646	72 ↓
<b>ET apical LPS stimulation</b>	Caco2 + LPS(-/+) /M1	649	72 ↓
	Caco2 + LPS(-/+) /M2	781	87 ↓
	Caco2 + LPS(+/-) /M1	66	73 ↓
	Caco2 + LPS(+/-) /M2	792	88 ↓
<b>ET basolateral LPS stimulation</b>	Caco2 /M1+ LPS(-/+)	624	69 ↓
	Caco2 /M2+ LPS(-/+)	778	87 ↓
	Caco2 /M1+ LPS(+/-)	685	76 ↓
	Caco2 /M2 + LPS(+/-)	773	86 ↓

TEER measurements unit =  $\Omega/\text{cm}^2$  and ↓ = decrease in % compared to untreated Caco2 cells.

**Table 5.2-2: Caco2 and MΦ subsets exhibit different cytokine profiles in response to K12-LPS, TNFα and IL-1β stimulation**

stimulus	secreted cytokine	Caco2		M1-like MΦs		M2-like MΦs	
LPS	TNFα	↑	**	↑	***	↑	**
	IL-1β	↑	*	↔	ns	↑	**
	IL-6	↑	**	↑	***	↑	***
	IL-10	↑	*	↑	***	↑	**
TNFα	TNFα	NA		NA		NA	
	IL-1β	↔	ns	↑	**	↑	*
	IL-6	↑	*	↑	**	↑	**
	IL-10	↑	**	↑	**	↑	***
IL-1β	TNFα	↑	***	↑	***	↔	ns
	IL-1β	NA		NA		NA	
	IL-6	↔	ns	↑	**	↑	**
	IL-10	↑	***	↔	ns	↑	*

↓ = decrease, ↑ = increase and ↔ = ns/ \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 and ns = not significant.

**Table 5.2-3: Cytokine production by co-culture models, Caco2/MΦ subsets, in homeostasis and inflammatory environment.**

a)

secreted cytokine	Homeostatic condition				Inflammatory condition			
	Apical LPS stimulation				Basolateral LPS stimulation			
	Caco2/M1	Caco2/M2	Caco2/M1	Caco2/M2	Caco2/M1	Caco2/M2	Caco2/M1	Caco2/M2
	Apical surface	Basolateral surface	Apical surface	Basolateral surface	Apical surface	Basolateral surface	Apical surface	Basolateral surface
TNFα	↔ ns	↓ ***	↔ ns	↓ **	↓ *	↓ ***	↓ ***	↓ **
IL-1β	↔ ns	↓ ***	↓ **	↓ **	↔ ns	↔ ns	↔ ns	↓ **
IL-6	↓ **	↔ ns	↔ ns	↔ ns	↔ ns	↔ ns	↓ ***	↑ *
IL-10	↔ ns	↓ *	↓ **	↓ ***	↔ ns	↓ ***	↓ **	↓ ***

b)

secreted cytokine	Inflammatory condition			
	Basolateral LPS stimulation			
	Caco2/M1	Caco2/M2	Caco2/M1	Caco2/M2
	Apical surface	Basolateral surface	Apical surface	Basolateral surface
TNFα	↓ **	↑ ***	↓ *	↔ ns
IL-1β	↔ ns	↓ *	↑ ***	↔ ns
IL-6	↔ ns	↓ *	↓ ***	↑ *
IL-10	↔ ns	↑ *	↓ *	↓ **

Table (a) show a compression of cytokine production between untreated and stimulus activated co-culture model in both homeostatic and inflammatory status. Table (b) show a compression of cytokine production in inflammatory status compared to homeostatic status. ↓ = decrease, ↑ = increase and ↔ = ns/ \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 and ns = not significant.

**Table 5.2-4: Differential sensitivity of LPS-induced co-culture model cytokines to TNF $\alpha$  and IL-1 $\beta$ .**

Apical TNF $\alpha$ stimulation					Apical TNF $\alpha$ stimulation + Basolateral LPS stimulation			
a)	Caco2/M1		Caco2/M2		Caco2/M1		Caco2/M2	
secreted cytokine	Apical surface	Basolateral surface	Apical surface	Basolateral surface	Apical surface	Basolateral surface	Apical surface	Basolateral surface
IL-1 $\beta$	↓ *	↑ **	↑ *	↔ ns	↔ ns	↔ ns	↑ **	↑ ***
IL-6	↔ ns	↔ ns	↔ ns	↔ ns	↔ ns	↔ ns	↑ **	↔ ns
IL-10	↑ *	↑ **	↑ **	↑ **	↑ *	↑ **	↑ **	↑ *

b)	Apical IL-1 $\beta$ stimulation				Apical IL-1 $\beta$ stimulation + Basolateral LPS stimulation			
	Caco2/M1		Caco2/M2		Caco2/M1		Caco2/M2	
	Apical surface	Basolateral surface	Apical surface	Basolateral surface	Apical surface	Basolateral surface	Apical surface	Basolateral surface
IL-1 $\beta$	↓ **	↑ **	↓ **	↔ ns	↔ ns	↑ ***	↓ **	↑ ***
IL-6	↔ ns	↔ ns	↔ ns	↑ *	↔ ns	↔ ns	↑ *	↔ ns
IL-10	↔ ns	↔ ns	↔ ns	↑ **	↔ ns	↔ ns	↑ **	↔ ns

↓ = decrease, ↑ = increase and ↔ = ns/ \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 and ns = not significant.

**Table 5.2-5: Effects of endotoxin tolerisation on co-culture model in homeostatic and inflammatory state.**

secreted cytokine	Endotoxin tolerisation Apical LPS stimulation				Endotoxin tolerisation Basolateral LPS stimulation			
	Caco2/M1		Caco2/M2		Caco2/M1		Caco2/M2	
	Apical surface	Basolateral surface	Apical surface	Basolateral surface	Apical surface	Basolateral surface	Apical surface	Basolateral surface
TNF $\alpha$	↓ **	↓ **	↔ ns	↔ ns	↓ *	↓ ***	↓ ***	↓ ***
IL-1 $\beta$	↔ ns	↓ *	↓ *	↓ *	↔ ns	↓ **	↔ ns	↓ *
IL-6	↑ **	↓ *	↓ ***	↔ ns	↔ ns	↑ **	↑ **	↓ *
IL-10	↔ ns	↑ **	↔ ns	↓ **	↓ *	↑ *	↑ *	↑ **

↓ = decrease, ↑ = increase and ↔ = ns/ \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 and ns = not significant.

### 5.3 Discussion

To mimic a complex interaction of the epithelium with nearby immune cells in the intestinal mucosa, we established epithelial-immune cell co-culture system, demonstrating a role for MΦ subsets in initiating and regulating tolerance and inflammation of epithelial cells. Caco2 cells have been widely used to study intestinal epithelial barrier function. However, these cells are cancer derived and not hypothetical to reflect the pathophysiological modifications in the case of inflammation. The complexity of the tissue *in vivo* is another point to be taken into attention. Immune cells are predominantly vital in the pathogenesis of inflammatory bowel disease as they are very dysregulated take up harmless non-pathogenic intestinal flora, processing them as an antigen (Van Deventer, 2002). Macrophages are able to consume some microbes or infected/ cancerous cells. Subsequently processing

they also present the antigen to helper T-cells, consequently activating adaptive immune responses. Several studies were published using a combination of different cell types (co-culture model), focussed on the immunomodulatory effects of probiotics into a range of gut mucosa such as DCs and epithelial cells. Therefore, the objective of this study was to expand on the cell characteristic and establish a model of intestinal mucosa in the state of both homeostasis and inflammation. This was accomplished by stimulating intestinal epithelial cells with pro-inflammatory compounds such as LPS from intestinal microflora and several cytokines such as TNF $\alpha$  and IL-1 $\beta$ . The current study was designed to determine the immunomodulation of different stimuli (K12-LPS, TNF $\alpha$  and IL-1 $\beta$ ) on epithelial cell barrier function (cytokine production and TEER) and M $\Phi$  subset roles in two status; normal/homeostatic and chronic inflammation. Experiments for stimulation of homeostasis were conducted by addition of LPS to the apical surface, whereas for induction of inflammation were conducted by addition of either TNF $\alpha$  or IL-1 $\beta$  to apical side and/or LPS to basolateral surface.

LPS, TNF or IL-1 $\beta$  could alter or disturb the barrier function of intestinal epithelia resulting in increased permeability or disrupted tight junctions (TJs). The effects of these stimuli on the TJs permeability of Caco2 monolayer were first determined by TEER measurement. The results suggested that TNF $\alpha$  is the most stimulus that able to alter the barrier of Caco2 whereas LPS showed less disruption.

Previously (in chapter 3) this study has shown that K12-LPS, TNF $\alpha$  and IL-1 $\beta$  have the potential role to initiate innate immune responses in M $\Phi$  subsets M1- and M2-like. In the present study, the Caco2 cell line is differentially sensitive to a range of stimuli. To illustrate, in response to IL-1 $\beta$ , Caco2 secreted TNF $\alpha$  100% higher than LPS stimulation, whereas Caco2 produce IL-1 $\beta$  and IL-6 more upon LPS stimulation. However, Caco2 did

not secrete IL-10 upon LPS stimulation, whereas TNF $\alpha$ -induced IL-10 by Caco2 cells was significantly higher than IL-1 $\beta$ -induced IL-10 (Figure 5.2-2). In comparison to monoculture experiment, this study showed immunosuppressive effects of Caco2 cells on *E. coli* LPS-stimulated M $\Phi$ s. Similar suppression was shown on *E. coli* LPS-stimulated Caco2 in presence of M $\Phi$ s. The secretion of the pro-inflammatory cytokines TNF $\alpha$ , IL-6 and, to some extent, IL-1 $\beta$  was significantly suppressed compared to monoculture results. On the other hand, anti-inflammatory cytokine, IL-10, which can suppress the synthesis of pro-inflammatory cytokines (de Waal Malefyt et al., 1991, Fiorentino et al., 1991), was not significantly affected by the presence of Caco2 cells with M1-like M $\Phi$ s, while it significantly increased in Caco2/M2-like M $\Phi$ s (Figure 5.2-4 and Figure 5.2-5). The use of semi-permeable transwell inserts, which prevented direct contact between Caco2 cells and M $\Phi$ s, suggesting that immunosuppressive factors were secreted from Caco2 cells. Previous studies exhibited that IEC profoundly inhibit monocyte activation (Haller et al., 2002, Nathens et al., 1995) and T-cell function (Christ et al., 1997) through both secreted mediators and cell–cell interactions. However, from the present experiments, the factor responsible for the suppression of TNF $\alpha$ , IL-1 $\beta$  and IL-6 synthesis remains unknown. Evidence had shown that chronic inflammatory damage in IBD is highlighted by a weakness in barrier function of the intestinal epithelium as well as dysregulation of intestinal mucosal immunity (Deuring et al., 2013). However, these results can be analysed from different angle instead of comparing this results to the monoculture results (controls), the comparison could be done between the two ways of treatment for the co-culture model where LPS was added either to apical side (homeostasis) or to basolateral side (inflammation). Interestingly, in the inflammatory environment, Caco2/M1-like M $\Phi$ s (inflammatory Model) (Figure 5.2-6) exhibited significant up-regulation of the pro-inflammatory cytokines, TNF $\alpha$  and IL-1 $\beta$ . Similar



observation about the significant upregulation of pro-inflammatory cytokines TNF $\alpha$  and IL-1 $\beta$  was obtained upon the combination stimulation of pro-inflammatory cytokines with LPS (Figure 5.2-8 and Figure 5.2-10). These data reflect the nature of pro-inflammatory M1 M $\Phi$  in the initiation the inflammation.

The production of IL-6 cytokine, in these results, can explain its dual function in pro- and anti-inflammatory role. IL-6 applies its anti-inflammatory effects through induction of SOCS proteins and STAT-3 activation (Xing et al., 1998). Certainly, SOCS3 is related with M1 M $\Phi$ s polarisation and is suppressive to anti-inflammatory signal and expression of IL-6 and IL-10. In opposition, SOCS3 expression knockdown M2 polarisation (Liu et al., 2008). Consequently, the mutual relationship between STAT-3 and SOCS3 would seem to regulate pro- or anti-inflammatory consequence of IL-6 and the M $\Phi$  polarisation between M1 and M2 effector subsets.

In contrast, IL-1 $\beta$  high production by Caco2/M2-like co-culture in the mentioned figures could facilitate anti-inflammatory responses through its ability to induce IL-10 expression (Foey et al., 1998); certainly, consequences from this research are suggestive of a positive association between IL-1 $\beta$  and IL-10, as these cytokines are produced strongest by the anti-inflammatory Caco2/M2-like model. Moreover, IL-1 $\beta$  production has been verified to be negatively related with the pro-inflammatory IKK $\beta$ -dependent NF-kB pathway (Greten et al., 2007); indicative of a non-pro-inflammatory character for IL-1 $\beta$  and the complex process of the NF-kB pathway in defining cell effector phenotype (Foey, 2014). In addition to the fact that IL-10 has a certain ability to suppress pro-inflammatory signaling, it also conserve intestinal epithelial barrier function (Jarry et al., 2008). Interestingly, the production of IL-10 upon LPS stimulation

was not significant, but it was induced significantly high in the inflammatory environment particularly upon TNF $\alpha$  stimulation.

Similar to results obtained in chapter 4, K12-LPS was able to induce endotoxin tolerisation to these two distinct models. In other words, M $\Phi$  subsets in combination with Caco2 cell line, as co-culture, exhibited endotoxin tolerisation similar to ET induced by mono-cultured M $\Phi$ s. ET induced by *E. coli* K12-LPS failed to demonstrate a differential subset-specific response. K12-LPS tolerised Caco2/M1- and Caco2/M2-like M $\Phi$ s exhibited down-regulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory cytokine, IL-10, which plays a protective role following endotoxin challenge. IL-10 level was only decreased in Caco2/M2 supernatant where ET was induced to apical side. However, as discussed previously, the M2-like M $\Phi$ s exhibited endogenous or bond membrane IL-10. Endogenous IL-10 assembly might be a part of the of endotoxin tolerisation.

The importance of these data in the context of IBD is difficult to interpret. In overall, encouraging tolerance by any mechanism is possible to be beneficial to chronic pathologies that result from over initiation of immune responses. These data obviously establish a role for *E. coli* LPS in tolerance induction M $\Phi$  subsets. It is speculated that endotoxin tolerance is important in maintaining innate immune homeostasis and that an impaired tolerance to LPS is associated with IBD.

Although this model displayed some characteristic and pathological variations, the reproducibility is difficult since the induced inflammatory effects depend on the dose, species and strain of the animal used. Caco2 cell lines were used as models to obtain significant figures about IEC involvement in the immune response of the mucosal intestine, particularly to assess the pathology of entropathogens (Kagnoff and

Eckmann, 1997, Jung et al., 1995). The present model cannot mimic the all processes within the GALT leading to specific immunity as some tissue specific cells exist in the GALT such as dendritic cells, intraepithelial lymphocytes, lamina propria lymphocytes, mast cells are missing. However, the model allows the validation of Caco2/MΦs interaction and representative two dimensional (2D) cell model for investigation, whose results will justify and rationalised the development of multicellular 3D models more representative of both the healthy/homeostatic and the inflammatory gut mucosal tissue.

## **Chapter 6: General discussion and conclusions**

## 6.1 General discussion

Three lines in this research were designed (monoculture of macrophage subsets, monoculture of Caco-2 epithelial cells, and co-culture models of Caco-2/M1 or Caco-2/M1, (Figure 1.3-1) to build up a good platform of knowledge about mucosal MΦ subsets characters in homeostasis and inflammatory.

### ***E. coli* K12-LPS differentially modulate MΦ subsets in a homeostatic or an inflammatory environment.**

This study aims to explore the gut mucosal MΦ subsets roles in homeostatic and inflammatory environment using a monocytic THP-1 cell line. It might be a disadvantage where the use of cell lines outside their usual environment because it might showed different sensitivity and responses compared with standard cells in the body. However, according to the homogeneous genetic background, which minimises the level of variability in the cell phenotype, which eases reproducibility of conclusions, THP-1 cells have some advantages over recently isolated PBMCs. It is essential to appreciate that differentiated THP-1 macrophages are different from macrophages derived from PBMCs, for example the regulation of apoptosis (Rogers et al., 2003). The THP-1 cell line is commonly used to study immune responses since of their ability to be differentiated into MΦ-like phenotype upon treatment by PMA and Vit.D<sub>3</sub> (Auwerx, 1991, Chanput et al., 2010, Schwende et al., 1996). The similarities between THP-1 derived MΦs and human PBMCs derived MΦs were shown in a number of publications with respect to several features such as polarizing ability (plasticity), macrophage morphology and adherence (Tsuchiya et al., 1982), the expression of macrophage

receptors (CD14, CD11b and CD36) (Schwende et al., 1996, Daigneault et al., 2010) and MΦ functions like phagocytosis, antigen presentation and accumulation of lipids (Gupta et al., 2005). It was established that THP-1 cells deliver a valid cell model system for assessing the response of macrophages to LPS (Sharif et al., 2007). In this investigation, THP-1 monocytes were differentiated into M1- or M2-like MΦs in order to use them for showing possible immune-regulation of K12-LPS to either macrophage monoculture (chapter 3 and 4) or macrophages/epithelial cells in co-culture models (chapter 5). Data in chapter 3 showed that macrophage subsets, which were generated using pro-monocytic THP-1 differentiation towards MΦs with M1-like and M2-like using PMA and vitamin D<sub>3</sub>, respectively, are differentially sensitive to challenging PAMPs. The profile of pro-inflammatory cytokine production induced by PAMPs is dependent on MΦ differentiation pathway. In general, M1-like MΦs were characterised as TNFα<sup>hi</sup>, IL-1β<sup>lo</sup>, IL-18<sup>hi</sup> and IL-6<sup>hi</sup> whereas, M2-like MΦs were TNFα<sup>lo</sup>, IL-1β<sup>hi</sup>, IL-18<sup>lo</sup> and IL-6<sup>lo</sup>. Apart from IL-18 profile, these data is supported by previous published data from our laboratory (Foey and Crean, 2013). The production of IL-18 by THP-1 derived MΦ subsets added extra validation markers for THP-1 differentiation by PMA and vitamin D<sub>3</sub>. From this platform, further experiments were applied to investigate more about mucosal MΦ behaviour in chronic inflammatory status and in the phenomenon of endotoxin tolerisation.

MΦ subsets showed further comparable features related with their regulation function. To illustrate, in chapter 3, long-term treatment with the pro-inflammatory cytokine, TNFα, was used to mimic a chronic inflammatory environment. No data was found in the literature on the question of how MΦ subsets can respond to TNFα stimulation. However, the long-term effects of TNF in disease associated with T cells was well

established (Cope et al., 1994). The results showed the ability of pro-inflammatory M1-like MΦs to initiate and increase inflammation upon TNFα-LPS-stimulation by producing more pro-inflammatory cytokines (TNFα and IL-18). On the other hand, under the same condition, M2-like MΦs suppressed pro-inflammatory cytokine production and increased anti-inflammatory cytokine production (IL-6 and IL-10). This helps to highlight the reparative and regulatory nature of M2 MΦ. Whereas M1 MΦs are normally considered the predominant MΦ involved in pathogen killing. Moreover, although M2 MΦs can produce TNFα, IL-1β, IL-6 and IL-10, these cytokines are less strongly related with the M2 phenotype (Foey, 2014, Mills and Ley, 2014, Baay et al., 2011). It was reported that TNFα, IFN-γ, LPS and GM-CSF are a group of stimuli that are mainly associated with M1 MΦs whereas the M2 stimulus group includes different stimuli that length different levels of recognition and response (Martinez and Gordon, 2014). Mucosal MΦs drive tolerogenic features in a homeostatic environment, while, keeping an effective phagocytic reaction at the same time. The homeostatic mucosal tolerance that associated with M2-like phenotype are dominated by the production of anti-inflammatory cytokines (TGFβ and IL-10) and phagocytic scavenger receptors (CD36, CD68 and CD206) (Smith et al., 2005, Platt and Mowat, 2008). It was reported that mucosal MΦs, in homeostatic tolerogenic state, produce TGFβ and IL-10, but fail to produce the pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, IL-8, IL-12, IL-18 and IL-23. Alongside the expression of the regulatory molecules CD33, CD200R and TGFβRI/RII, this suppression is more reflected by the lack of expression of the pro-inflammatory molecule, TREM-1, CD14/TLRs, FcRs and costimulatory molecules (CD40, CD80, CD86) (Foey, 2015, Foey, 2012). However, with respect to inflammatory diseases such as Crohn's disease and Chronic periodontitis and increased in immune suppression – related diseases such as colorectal cancer and oral squamous cell

carcinoma, this tolerogenic/homeostatic role of MΦs is dysregulated and mucosal tolerance is broken. These pathologies display mechanisms associated to M1- or M2-driven responses. MΦs show an inflammatory phenotype, which is comparable to the M1 MΦ subset in the situation of pro-inflammatory diseases. This inflammatory state leads to M1-like MΦ activation/ differentiation with the parallel pro-inflammatory cytokine up-regulation and co-stimulatory molecule expression (Bouma and Strober, 2003). Therefore, the pro-inflammatory MΦs produce/secrete a wide variety of effector molecules that include: PRRs (CD14, TLR2, TLR4, TLR5), chemokine receptors (CCR5, CXCR4), FcRs (CD16, CD32, CD64, CD89), and the pro-inflammatory markers/cytokines (TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-18, TREM-1 and CCL20) HLA-DR and CRs (Foey, 2012, Rogler et al., 1999, Smythies et al., 2005). Perhaps the most compelling note of this study is the fact that, upon long-time TNF $\alpha$  stimulation prior to LPS challenge, M1-like MΦs increase inflammation by producing pro-inflammatory cytokines whereas M2-like show suppression/regulation roles by producing anti-inflammatory cytokines. Consequently, more research aiming M1 MΦs therapeutically or indeed the increase of M2-mediated reactions can characterise a realistic treatment in the control of this chronic inflammatory disease.

As mentioned earlier, in this investigation THP-1 derived macrophage subsets differentially secreted IL-1 $\beta$  and IL-18 upon K12-LPS stimulation. IL-1 $\beta$  and IL-18 cytokines are associated with inflammasome activity where PAMPs (LPS) trigger assembly of the intracellular innate immune sensor Nlrp3, causing caspase-1 activation and production of pro-inflammatory cytokines; IL-1 $\beta$  and IL-18 (Dinarello et al., 2013b). The precise mechanism of the Nlrp3 inflammasome activation and the subsequent activation of caspase-1 is unclear. In chapter 3, it has been shown that IL-1 $\beta$  and IL-18



production in M1-like MΦs was caspase-1-dependent while, IL-1β only was caspase-1 dependent in M2-like MΦs. However, activation of caspase-1 is just one mechanism for cleavage of IL-1β and IL-18. Therefore, the reality of alternative pathways of IL-β and IL-18 activation that are independent of the inflammasome should be acknowledged. One of the most interesting results in this study is IL-1β / M2-like MΦs production and responsiveness. To illustrate, it is remarkable that (chapter 3/5) M2-like MΦs were a good producer of IL-1β but on the other hand, they did not response to IL-1β stimulation. More studies need to be applied to explore the mechanism of the recognition of IL-1β cytokine by MΦ subsets. Taken together the results from chapter 3 with data from the literature, it can be accomplish that THP-1 derived MΦ subsets cells seem to represent a basic, reliable and suitable model to explore macrophage polarisation, macrophages roles/responses, and possible their immunomodulatory effects.

#### **Endotoxin tolerisation induced in MΦ subsets in a homeostatic or an inflammatory environment.**

Besides the influence of differentiation signals and polarising activation, MΦ division to different subsets is possible to be firmed by suppressive signals or tolerisation. ET was first termed by the observation that LPS pre-stimulation suppressed innate immune cells response to activation upon LPS retreatment. Subsequently, ET has been presented in MΦs for a range of pro-inflammatory cytokine such as TNFα and IL-1β and TLR-mediated signals include LPS, LTA, PGN, and Flagellin (Biswas and Lopez-Collazo, 2009). MΦ functionality suppression might benefit infectious microbes but at the same time positively prevent damaging inflammatory responses, therefore allowing for a good environment for the pathogen to recover its numbers through

growth. In the case of the gut pathogen *Escherichia coli*, associated with wide variety of intestinal diseases, such as Crohn's disease and neonatal meningitis (Martinez - Medina et al., 2009). Both MΦ subsets were sensitive to K12-LPS-induced ET, where M1- and M2-like MΦ subsets displayed down-regulation of pro-inflammatory cytokines and no significant change was shown in anti-inflammatory cytokine production, IL-10, by both M1- and M2-like MΦs. It remains to be find out if ET involves a single critical signaling pathway or serial multiple changes in signaling events during tolerance induction. It was suggested that many endogenous suppressors exist can suppress TLR-mediated activatory or polarising responses. The endogenous suppressors include Tollip, IRAK-M, SIGIRR. Interestingly, not all signaling pathways are down-regulated and some proteins are up regulated. In this investigation, Tollip was more associated with M1-like MΦ whereas IRAK-M and SIGIRR were more associated with M1-ike MΦs (chapter 4). The likely reason is that up-regulation of Tollip and SIGIRR expression in M1s, IRAK-M expression in M2s is not a unique mechanism, and, obviously, there are other additional regulatory mechanisms to control the response to LPS. These include MD2, Myd88s, TRIAD3A, and several further suppressive molecules show a role in regulating MΦ responses such as CD200R, CD47/SIRP1α, Siglecs 3-10 and CD32 reviewed in (Foey, 2015). However, ET may be reflective of action suppression or might modify polarisation status of the MΦ from M1 to M2. The amplification of such regulatory molecules could characterise a realistic method in controlling chronic inflammatory diseases such as Crohn's disease and chronic periodontitis. Further studies need to be done on the effect of ET on cellular signaling, mediator production, and transcription factors. Therapeutic interference will be dependent on amplification of MΦ polarisation plasticity, MΦ subset precise ET

mechanisms and downstream effects on polarisation of T cell reactions (Foey and Crean, 2013).

***E. coli* K12-LPS differentially modulate Intestinal epithelial barrier function in a homeostatic or inflammatory environment.**

The intestinal epithelium functions as vital cell barrier between the gut lumen and the lamina propria. It is recognised for its strength against attack of luminal bacteria that may be accrue with bacterial cell density of more than  $10^{14}$  CFU/ml. This barrier typically does not respond to commensal bacteria, but triggers the pro-inflammatory signaling pathway in the response to pathogenic bacteria. Conversely, in IBD, together with an augmented permeability, the barrier fails to achieve such initial recognition, perhaps as a result of lack of defence molecules, exhibiting no response before the bacteria attack the basolateral side (Elphick et al., 2008). The exact signaling procedures are still unclear. Previous *in vitro* investigations, targeting IBD, have been shown in typical mono-culture of cell lines. These cell lines characterise the normal mucosa with integral tight junctions and strong barrier features. Nevertheless, such a procedure does not necessarily reflect the pathophysiological modifications happening in an inflamed area of IBD. Furthermore, a single cell line can never describe the compound interaction of different cell types during an inflammation (Leonard et al., 2010).

Caco2 cell line are widely established as a model of normal, healthy intestinal mucosa. However, as this model consist only of enterocytes, it cannot mimic the complex connections with other cells particularly the immune system cells. These interactions

could be very important for the epithelial barrier role (Leonard et al., 2010). In chapter 5 of this study, THP-1 derived MΦs and Caco2 cells were used to develop *in vitro* co-culture system, which mimics the interaction of gut epithelial cells with immune cells (M2-like or M1-like macrophages) of normal homeostasis (Caco-2/M2) or chronic inflammation model (Caco-2/M1). THP-1 cell line and Caco-2 have been widely used to study intestinal epithelial barrier function. However, these cells are cancer-derived and not thought to reflect the pathophysiological modifications in the state of inflammation. Consequently, the objective of this study was to develop on the cell characteristic and establish a model of intestinal mucosa in the condition of homeostasis. This was done by stimulating intestinal epithelial cells with pro-inflammatory compounds such as LPS. However, the second object was to determine the role of pro- and anti-inflammatory MΦs in response to pro-inflammatory cytokines (TNFα and IL-1β) in the presence or absence of LPS in mono- and co-culture. TNFα and IL-1β overproduction by uncontrolled immune cells such as mucosal macrophage contributes to initiation of tissue harm in IBD (Strober and Fuss, 2011). The main idea of the use of TNFα and IL-1β in this experiment was to mimic a chronic inflammatory environment, which will allow exploring more of MΦ subsets functions. The co-culture itself displayed the estimated arrangement of Caco2 construction the multiple layer on top of the culture, while macrophages were settled in the bottom of the wells. Therefore, this situation appeared to be not perfect as in several areas we find immunocompetent cells generally located directly below the epithelial layer and even reaching out to the apical side of the model. Some studies assumed the result that antigen transfer through the intestinal barrier is conducted by specialised M-cells (Iwasaki, 2007). Therefore, it might be a good idea to design an experiment where Caco2 cells contact physically and directly with MΦ subsets although this co-culture

model was an efficient tool for narrowing the gap between animal testing and typical cell culture system and delivered a relevance of inflamed tissue, where it preserved the suitability of cell culture. Thus, transwell investigated the effect of soluble secreted signals. Direct cell-to-cell contact will go a step further in investigating physical contact signals between both cells, Caco2 and MΦ subsets.

Former studies showed that co-culture with Caco2 cells and triggered monocyte cell line THP-1 presented apoptotic and necrotic result to the Caco2 cells and damaging monolayer integrity. Another model has also been designed to mimic the respiratory tract using epithelial cells and immunocompetent cells to investigate the particle circulation (Rothen-Rutishauser et al., 2005). With regard to the inflammatory bowel disease model, there have been a couple of methods using primary blood cells from IBD patients and Caco2 or primary colonic crypt cells. This model has presented that cells from IBD patients displayed more IFN- $\gamma$  than the healthy model but might not show any pathophysiological significance since they are not cultured to gather the physiological procedure in the intestine, although cells were cultured together (Bisping et al., 2001). Consequently, the experiment was designed for this model, which, consists of the relevant cells for the inflammation and antigen presenting signaling, to allow controlling stimulation, and mimic major pathophysiological modifications happening in IBD.

Macrophage cells are involved in the initiation of CD according their ability to dysregulate pro- and anti-inflammatory cytokine production as a result of stimulation of endogenous and exogenous stimuli, microbial PAMPs and cytokines, (Butcher et al., 2005, Pull et al., 2005, Smith et al., 2011). Results, in this study, presented the pro-inflammatory M1-like MΦs ability to recruit and increase inflammation upon TNF $\alpha$ -

LPS-stimulation by producing more TNF $\alpha$  and IL-18 while M2-like M $\Phi$ s suppressed pro-inflammatory cytokine production and increased anti-inflammatory cytokine production, IL-6 and IL-10. It also showed that both M $\Phi$  subsets were sensitive to K12-LPS-induced ET and Tollip and SIGIRR were associated with M1-like M $\Phi$ s, whereas IRAK-M was associated with M2-like M $\Phi$ s.

Taking these findings together considering that M2-like M $\Phi$  is responsible to initiate homeostatic environment and the inflammatory environment, which is contributing to M1-like M $\Phi$  activation in the line where ET status is broken in the case of CD.

This subset-independent manner sensitivity to ET, coupled with the transmural skip-lesion-associated nature of this chronic inflammatory disease, is typically suggestive that inflammation or immune activation is tissue-destructive while tolerisation or immune suppression is of advantage to the host through ending the mechanisms of these tissue-destructive. Future therapeutic intervention will be reliant on clarification of M $\Phi$  polarisation plasticity, M $\Phi$  subset specific ET mechanisms and downstream effects on polarisation of T cells responses, which are involved in CD. Additionally, the therapeutic targeting of M1 M $\Phi$ s via the encouragement of the negative regulators augmentation (TOLLIP and SIGIRR) or certainly the development of M2-mediated responses or development a mechanism of IRAK-M recruitment and increase IL-10 production could represent a new treatment in the regulation of this chronic inflammatory disease.

## 6.2 Limitation and future work

Results in the research create a platform of knowledge about the polarisation and behaviour of PMA and Vitamin D<sub>3</sub> THP-1 differentiated MΦ subsets, M1-like and M2-like MΦ, respectively, and Caco-2 epithelial cells. Additionally, these cells were used to improve *in vitro* co-culture models while Caco-2 epithelial cells were co-cultured with M1-like MΦs, resembling a chronic inflammation model, and Caco-2 co-cultured with M2-like MΦs, resembling a model of normal gut homeostasis, were used to explore PAMPs modulation of macrophage behaviour and macrophage -influenced epithelial barrier function. Results in chapter 3 showed that MΦ subsets differentially sensitive to TNFα and IL-1β stimulation in present or absent of LPS. Further studies need to be applied on this stimulus receptor and signalling. In addition, this experiment could be redesigned to be applied on the co-culture models used in chapter 5. MΦ subsets differentially produced IL-18 and differentially produced and respond to IL-1β. The role caspase-1 is different between M1 and M2-like MΦs. Taking together, it is recommended to investigate more about inflammasome activation in MΦ subsets include inflammasome signaling cascade, NLRP3 initiation. The main finding in chapter 4 was the role of negative regulatory molecules in the induction of ET in MΦ subsets where TOLLIP and SIGIRR were more associated with M1-like and IRAK-M was associated with M2-like MΦs. It was suggested to do more study to confirm these results by using siRNA technique, a method of preventing translation of specific genes by interferes it with the expression with complementary nucleotide sequences by degrading mRNA after transcription (Agrawal et al., 2003). It is also recommended to investigate about the production of another anti-inflammatory cytokine in ET such as TGF-β. Results of IL-6 production by endotoxin tolerised-M2-like MΦs in parallel with of STAT-3 and SOCS-3 results in chapter 4 gave an indication to study the association between JAK/STAT

signalling pathway and ET in MΦ subsets. Furthermore, homo-tolerisation experiments showed interesting results especially the relation between K12-LPS and PGN, which ignited more ideas to be investigated. Finally, as the model in chapter 5 presents a validation of interaction of Caco2/MΦs and characterise two dimensional 2D cell model for study, these outcomes will restructure and rationalise the improvement of multicellular 3D models.

### 6.3 Conclusion

In conclusion, as this investigation has focused on the mucosal interactions in health and disease, further characterised M1-and M2-like MΦ subsets with respect to pro-inflammatory cytokine profile upon stimulation especially with *E. coli* K12-LPS. Firstly, a dichotomy in cytokine secretion was demonstrated where M1 MΦs are indeed the predominant pro-inflammatory cell. M1 MΦs, representative of pro-inflammatory pathological cells, were mainly pro-inflammatory and strongly produced  $\text{TNF}\alpha^{\text{hi}}$ ,  $\text{IL-1}\beta^{\text{lo}}$ ,  $\text{IL-18}^{\text{hi}}$ ,  $\text{IL-6}^{\text{hi}}$  and secreted  $\text{IL-10}^{\text{hi}}$ , whereas M2 subset, representative of regulatory, anti-inflammatory cells, was indeed a low-level producer of  $\text{TNF}\alpha^{\text{lo}}$ ,  $\text{IL-1}\beta^{\text{hi}}$ ,  $\text{IL-18}^{\text{lo}}$ ,  $\text{IL-6}^{\text{lo}}$  and secreted  $\text{IL-10}^{\text{lo}}$ . Furthermore, in the chronic inflammation (long term  $\text{TNF}\alpha$  stimulation), M1-like MΦs increased the inflammation by production of pro-inflammatory cytokines such as  $\text{TNF}\alpha$ , in the presence of LPS, whereas M2-like MΦs, representative of homeostasis, showed remarkable tolerance condition with high level of anti-inflammatory cytokines, such as IL-10. However, MΦ subsets showed similar sensitivity to inflammation induced by  $\text{IL-1}\beta$ . Additionally, with regards to the inflammasome activity in MΦ subsets,  $\text{IL-1}\beta$  and IL-18 secretion was dependent on the activation of caspase-1 pathway in M1-like MΦs, whereas only  $\text{IL-1}\beta$  production was caspase-1 dependent in M2-like MΦs.



Secondly, upon investigation of sensitivity of these subsets to tolerisation, it was observed that both subset were sensitive to *E. coli*-induced suppression of pro-inflammatory cytokines. This cytokine profile is likely to be associated with the up-regulation of TLR4 and Tollip in M1-like MΦs, whereas it might be as a consequence of IL-10 and IRAK-M up-regulation. This would suggest that such mechanisms of ET might be beneficial for survival and immunopathological mechanisms driven by the pathogen. It was also established that comparing to M1-like MΦs; M2-like MΦs differently produce IL-10 cytokine along with an endogenous cell-associated activity. However, both M1 and M2 MΦ were sensitive to the anti-inflammatory effects of IL-10 in the present of *E. coli* K12-LPS. M1- and M2-like MΦs were able to performed homo- and hetero-tolerisation induced by different PAMPs.

Finally, in this study, epithelial–macrophages cells co-culture system was established and the role for MΦ subsets in initiating and regulating tolerance and inflammation of epithelial cells was demonstrated. LPS, TNF and IL-1β stimulation was able to disturb the barrier function of intestinal epithelia resulting in increased permeability or disrupted tight junctions TJs as TNFα was the most stimulus that showed disruption. In the present study, the Caco2 cell line is differentially sensitive to a range of stimuli i.e. more sensitive to pro-inflammatory cytokines, TNFα and less sensitive to LPS. In comparison to monoculture experiment, the co-culture system showed immunosuppressive effects of Caco2 cells on *E. coli* LPS-stimulated MΦs. Similar suppression was shown on *E. coli* LPS-stimulated Caco2 in presence of MΦs. To illustrate, Caco2/M1-like MΦs (inflammatory Model) displayed significant up-regulation of the pro-inflammatory cytokines, TNFα and IL-1β in the inflammatory environment, which reflect the nature of pro-inflammatory M1 MΦ in the initiation

the inflammation. Interestingly, in homeostatic state, K12-LPS induced suppression of inflammatory cytokines in M1-like subset in co-culture model whereas M2-like subset in Caco2/M2 model was refractory to tolerisation. However, in the inflammatory condition, K12-LPS induced ET in both subsets in the co-culture system.

To conclude, any future investigation on MΦ subset tolerisation can be achieved realistically upon a full understanding of the immunopathological pathways behind such inflammatory diseases as CD and by development of multicellular 3D models more representative of both the healthy/homeostatic and the inflammatory gut mucosal tissue.

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# Appendices



# Appendix 1

## General material

### 1 Tissue culture reagents and material

Table 1-1: Tissue culture reagents and material

Product	Product code	Supplier
1, 25-(OH) <sub>2</sub> Vitamin D <sub>3</sub>	D1530	Sigma-Aldrich, Poole, UK
Bovine serum albumin (BSA)	A8531-1VL	Sigma-Aldrich, Poole, UK
Caco-2 cell line		ECACC, Salisbury, UK
Caspase-1/ICE Inhibitor Z-WEHD-FMK	FMK002	Bio-Techne Ltd, Abingdon, UK
Cell culture costumes	(Flasks, plates. Tips, pipettes)	Greiner Bio-one-Ltd, Stonehouse, UK
DMEM	BE12-614F/12	Lonza Bioscience ltd, UK.
Dulbecco's Phosphate Buffered Saline	D5652-10X1L	Sigma-Aldrich, Poole, UK
dimethyl sulfoxide (DMSO)	D8418	Sigma-Aldrich, Poole, UK
<i>Escherichia coli</i> K12-LPS	t1rl-pek1ps	Invivogen, UK
Ethyl alcohol	E7023	Sigma-Aldrich, Poole, UK
Foetal Calf Serum	FCS-SA-10545/500	Labtech International

		Limited, East Sussex, UK
IL-10 standard antibody	93/722	NIBSC, Hertfordshire, UK
IL-1 $\beta$ standard antibody	86/680	NIBSC, Hertfordshire, UK
L-Glutamine 200nM		Fisher Scientific UK Ltd
Penicillin/streptomycin	A 2213	Source Bioscienc
Peptidoglycan (PGN)	77145	Sigma-Aldrich, Poole, UK
phorbol-12-myristate acetate (PMA)	P1585	Sigma-Aldrich, Poole, UK
Purified anti-human anti-IL-10	501402	Biolegend, London, UK
Purified Rat IgG1, $\kappa$ Isotype Ctrl Antibody	400402	Biolegend, London, UK
RMPI 1640	12-167F	Lonza Bioscience Ltd, UK.
THINCERT CELL CULTURE INSERT-WELL PLATES	665640	Greiner Bio-one-Ltd, Stonehouse, UK
THP-1 cells line		ECACC, Salisbury, UK
TLR2-ligand, lipoteichoic acid (LTA)	tlrl-pslta	InvivoGen, UK
TNF $\alpha$ standard antibody	12/154	NIBSC, Hertfordshire, UK
Ultrapure <i>Porphyromonas gingivalis</i> LPS	tlrl-ppglps	InvivoGen, UK
Cryopreservation Storage media		

20%v/v FBS/10% v/v dimethyl sulfoxide (DMSO)/ DMEM or RPMI

## 2 ELISA

**Table 2-1: ELISA reagents and Antibodies.**

Product	Product code	Supplier
Bovine serum albumin (BSA)	A8531-1VL	Sigma-Aldrich, Poole, UK
IL-10 Purified Rat Anti-Human capture antibody	554497	BD Pharmingen, UK
IL-10 Biotin Anti-Human detect antibody	554499	BD Pharmingen, UK
IL-1 $\beta$ capture antibody	MAB601	R&D Systems, Abingdon, UK
IL-1 $\beta$ detection antibody	BAF201	R&D Systems, Abingdon, UK
IL-1 $\beta$ standard antibody	1989	NIBSC, Hertfordshire, UK
IL-6 Purified Rat Anti-Human capture antibody	554543	BD Pharmingen, UK
IL-6 Biotin Anti-Human detection antibody	554546	BD Pharmingen, UK
IL-6 standard antibody	89/548	NIBSC, Hertfordshire, UK
IL-8 Purified Rat Anti-Human capture antibody	554716	BD Pharmingen, UK
IL-8 Biotin Anti-Human detection	554718	BD Pharmingen, UK

<b>antibody</b>		
<b>IL-8 standard antibody</b>	<b>89/520</b>	<b>NIBSC, Hertfordshire, UK</b>
<b>L-18 Human Matched Antibody Pair</b>	<b>BMS267/2MST</b>	<b>eBioscience, Thermo Fisher Scientific Inc, UK</b>
<b>Nunc ELISA Plates Immunosorbent.</b>	<b>10547781</b>	<b>Fisher Scientific, UK</b>
<b>Phosphate buffered saline tablets</b>	<b>P4417</b>	<b>Sigma-Aldrich, Poole, UK</b>
<b>Purified anti-human IL-10 Antibody</b>	<b>501402</b>	<b>Biolegend, London, UK</b>
<b>Streptavidin HRP</b>	<b>DY998</b>	<b>R&amp;D Systems, Abingdon, UK</b>
<b>TMB microwell peroxidase</b>	<b>50-76-00</b>	<b>Insight Biotechnology</b>
<b>TNF<math>\alpha</math> anti-human capture antibody</b>	<b>551220</b>	<b>BD Pharmingen, UK</b>
<b>TNF<math>\alpha</math> Biotin Mouse Anti-Human detection antibody</b>	<b>554511</b>	<b>BD Pharmingen, UK</b>
<b>TNF<math>\alpha</math> standard antibody</b>	<b>12/154</b>	<b>NIBSC, Hertfordshire, UK</b>
<b>Tween 20</b>	<b>P2287-100ML</b>	<b>Sigma-Aldrich, Poole, UK</b>

### 3 Protein analysis

#### **Table 3-1: Western Blotting solutions**

Western Blotting reagents were obtained from

Sigma-Aldrich, Poole, UK

#### **Lysis Buffer**

##### **Ripa buffer**

Protease Inhibitor Cocktail

1:20 = for 200µl lysis buffer add 10µl

Phosphatase Inhibitor Cocktail

1:100 = for 200µl lysis buffer add 2µl

#### **SDS-PAGE reagents**

Acrylamide, electrophoresis grade

Bis- acryl amide (N,N –methylenebisacrylamide)

Tris (2-hydroxymethyl-2-methyl-1, 3-propanediol)

SDS (sodium dodecyl sulphate or sodium lauryl sulphate)

TEMED (N, N, N, N,-tertamethylene-ethylenediamine)

APS (Ammonium per sulphate)

2-mercaptoethanol

Glycerol

Bromophenol blue

Glycine

HCl (Hydrochloric acid)

#### **Solution A (separating gel buffer)**

Acrylamide Stock Solution, 100 ml 30% (w/v) acrylamide

(w/v) bis- acrylamide

**Solution B (separating gel buffer):**

1.125 M Tris base 68.14 g per 500 ml

0.3%w/v SDS 1.5 g per 500 ml

Dissolved in DW to 450 ml adjusted to pH 8.8 with HCl.

Top up to 500 ml with DW.

**Solution C (Stacking buffer)**

140 mM Tris 8.42 g per 500 ml

0.11%w/v SDS 0.55 g per 500 ml

Dissolve in DW to 450 ml. Adjust to pH 6.8 with concentrated HCl 100%. Top up to 500 ml with DW

10%w/v APS (0.1g APS dissolved into 1ml DW)

**Electrophoresis buffer (Running buffer)**

Tris 3 g

Glycine 14.4 g

SDS 1 g

Dissolved in DW 1L, pH 8.3

Transfer buffer stock (10X Exc. Methanol)

Tris base 30.3g

Glycine 144g

DW 1L.

**Transfer buffer working solution**

10X transfer buffer 100 ml

Methanol 200 ml

DW 700 ml

Washing solution

tris buffered saline with tween 20 (TBST)

TBS-Tween-20 (0.1% v/v)/TBS

10xTBS ( tris buffered saline)

NaCl 80g

Tris 24.4g

DW Mix in 800ml of DW, adjust to pH 7.6, and make up

to 1L with DW

TBS-Tween-20 (TBST)

10x TBS 40ml

DW 959ml

Tween-20 1ml

### **Blocking solution**

TBST 100 ml

5% w/v BSA 5g

### **Stripping buffer**

Mild stripping

15 g glycine

1 g SDS

10 ml Tween 20

Adjust pH to 2.2 Top up to 1 L with DW

**Table 3-2: Protein analysis reagents.**

<b>Product</b>	<b>Product code</b>	<b>Supplier</b>
<b>Anti-rabbit IgG, HRP-linked Antibody</b>	<b>7074P2</b>	<b>New England Biolabs, Herts, UK</b>
<b>Anti-SIGIRR antibody</b>	<b>ab22053</b>	<b>Abcam, Cambridge,UK</b>
<b>Biotinylated Protein Ladder Detection</b>	<b>7727S</b>	<b>New England Biolabs, Herts, UK</b>
<b>Bovine serum albumin (BSA)</b>	<b>A8531-1VL</b>	<b>Sigma-Aldrich, Poole, UK</b>
<b>Criterion Xt precast gel, 4-12% Bis-Tris</b>	<b>345-0123</b>	<b>Bio-Rad Laboratories Ltd, Hertfordshire, UK</b>
<b>Deoxynucleotide Mix,10mM</b>	<b>D7295-.5ML</b>	<b>Sigma-Aldrich, Poole, UK</b>
<b><i>Ethyl alcohol</i></b>	<b>E7023</b>	<b>Sigma-Aldrich, Poole, UK</b>
<b>Gel loading Tips</b>	<b>LW1100</b>	<b>Alpha Laboratories Ltd, Hampshire, UK.</b>
<b>Goat Anti-Rabbit IgG (H+L)-HRP Conjugate</b>	<b>172-1019</b>	<b>Bio-Rad Laboratories Ltd, Hertfordshire, UK</b>
<b>Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (100X</b>	<b>78442</b>	<b>Thermo Scientific, UK</b>
<b>IRAK-M Antibody</b>	<b>PA5-17473</b>	<b>Fisher Scientific - UK Ltd</b>
<b>Luminata Crescendo Western HRP substrate</b>	<b>WBLUR0100</b>	<b>Millipore</b>
<b>Micro BCA Protein Assay Kit</b>	<b>23235</b>	<b>Thermo Fisher Scientific, UK</b>



Pierce Prestained Protein MW Marker	26612	Thermo Fisher Scientific, UK
PVDF Transfer Membrane	88518	Thermo Fisher Scientific, UK
Rabbit anti-Mouse IgG (H+L) Secondary Antibody [Biotin]	NB720-B	R&D Systems, Abingdon, UK
Restore Western Blot Stripping Buffer	21062	Thermo Fisher Scientific , UK
RIPA Lysis and Extraction Buffer	89900	Thermo Fisher Scientific,UK
STAT-3 (79D7) Rabbit mAb	4904P	New England Biolabs, Herts, UK
TLR4 Antibody	NB100-56566	R&D Systems, Abingdon, UK
Tollip Antibody	4748	New England Biolabs, Herts, UK
Water ,Molecular Biology Reagent	W4502-1L	Sigma-Aldrich, Poole, UK
Western Blotting Filter Paper	88620	Thermo Fisher Scientific, UK

## 4 RNA qualification

**Table 4-4-1: RNA qualification reagents**

Product	Product code	Supplier
Bovine serum albumin (BSA)	A8531-1VL	Sigma-Aldrich, Poole, UK
Deoxynucleotide Mix,10mM	D7295-.5ML	Sigma-Aldrich, Poole, UK

RNA extraction and PCR costumes	(plates, Tips and pipettes)	Fisher Scientific UK
<i>Ethyl alcohol</i>	E7023	Sigma-Aldrich, Poole, UK
GenElute mammalian total RNA extraction kit	RTN350-1KT	Sigma-Aldrich, Poole, UK
Ladder 100 bp DNA ladder	15628019	Invitrogen-Gibco
M-MLV Reverse Transcriptase	M1302-40KU	Sigma-Aldrich, Poole, UK
On-Column DNase I Digestion Set	DNASE70	Sigma-Aldrich, Poole, UK
Phosphate buffered saline tablets	P4417	Sigma-Aldrich, Poole, UK
Primers		Eurofins Genomics, Wolverhampton, UK
Random Nonamers	R7647-100UL	Sigma-Aldrich, Poole, UK
Ribonuclease inhibitor human	R2520-2.5KU	Sigma-Aldrich, Poole, UK
SYBR® Green Master Mix	4472908	Applied Biosystems, Cheshire - UK
Water ,Molecular Biology Reagent	W4502-1L	Sigma-Aldrich, Poole, UK

**Table 4-4-2: Summary of primers used in this study and the amplicon product size**

Target	Forward primer 5'	Size(base pair bp)	Reverse primer 3'	Size(base pair bp)	Product size bp
<b>β-actin</b>	CCTGTACGCCAACACAGTGC	20	ATACTCCTGCTTGCTGATCC	20	211
<b>GAPDH</b>	CTGCTCCTCCTGTTTCGACAGT	21	CCGTTGACTCCGACCTTCAC	23	100
<b>TNFα</b>	ACATCCAACCTTCCCAAACG	20	GCCCCCAATTCTCTTTTGTAG	22	151
<b>IL-10</b>	AGGAGGTGATGCCCCAAGCT GA	22	TCGATGACAGCGCCGTAGCC T	21	110
<b>IL-6</b>	TGGCTGCAGGACATGACAAC	20	TGAGGTGCCCATGCTACATTT	20	100
<b>IL-1 β</b>	GCCAATCTTCATTGCTCAAGT	21	AGCCATCATTTCACTGGCGA	20	73

<b>TLR4</b>	AGCCCTTCACCCCGATTC	18	TAGAAATTCAGCTCCATGCAT TG	23	100
<b>TLR2</b>	GGCATGTGCTGTGCTCTGTT	20	GGAGCCAGGCCACATC	17	100
<b>Nod-2</b>	CAGAAATTTCAAACGGCCTCAC TA	23	ATGAAATGGAACTGCCTCTT GTG	23	102
<b>Tollip</b>	TCTCATGCCGTTCTGGAAAAT	21	TCACATCACAAAATGCCATGA A	22	110
<b>IRAK-M</b>	TTCAACCATGCTCGGTCATCT	21	CATACCAGGAGAACTACAGC AGA	23	137
<b>SIGIRR (1)</b>	GACCTGGCTTGTGTCTTTGT	20	GGGTGGGGTTTAGGGTTAGG A	21	94

# Appendix 2

## Published papers:

Foey, A.D., Habil, N., Al-Shaghhdali, K. and Crean, S.J. (2017): Porphyromonas gingivalis-stimulated macrophage subsets exhibit differential induction and responsiveness to interleukin-10. Archives of Oral Biology 73:282-288.

Archives of Oral Biology 73 (2017) 282–288

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Archives of Oral Biology

journal homepage: [www.elsevier.com/locate/aob](http://www.elsevier.com/locate/aob)

*Porphyromonas gingivalis*-stimulated macrophage subsets exhibit differential induction and responsiveness to interleukin-10

Andrew D. Foey<sup>a,\*</sup>, Neama Habil<sup>a</sup>, Khalid Al-Shaghhdali<sup>a</sup>, StJohn Crean<sup>b</sup>

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**ABSTRACT**

**Objectives:** Oral mucosal macrophages (Mφs) determine immune responses; maintaining tolerance whilst retaining the capacity to activate defences against pathogens. Mφ responses are determined by two distinct subsets; pro-inflammatory M1- and anti-inflammatory/regulatory M2-Mφs. Tolerance induction is driven by M2 Mφs, whereas M1-like Mφs predominate in inflammation, such as that exhibited in chronic *Porphyromonas gingivalis* (PG) periodontal infection. Mφ responses can be suppressed to benefit either the host or the pathogen. Chronic stimulation by pathogen associated molecular patterns (PAMPs), such as LPS, is well established to induce tolerance. The aim of this study was to investigate the *P. gingivalis*-driven induction of and responsiveness to the suppressive, anti-inflammatory cytokine, IL-10, by Mφ subsets.

**Methods:** M1- and M2-like Mφs were generated *in vitro* from the THP-1 monocyte cell line by differentiation with PMA and Vitamin D<sub>3</sub>, respectively. Mφ subsets were stimulated by PG-LPS in the presence or absence of IL-10.

**Results:** PG-LPS differentially induced IL-10 secretion and endogenous IL-10 activity in M1- and M2-like subsets. In addition, these subsets exhibited differential sensitivity to IL-10-mediated suppression of TNFα, where M2 Mφs were sensitive to IL-10 and M1 Mφs were refractory to suppression. In addition, this differential responsiveness to IL-10 was independent of IL-10-binding and expression of the IL-10 receptor signal transducing subunit, IL-10Rβ, but was in fact dependent on activation of STAT-3.

**Conclusion:** *P. gingivalis* selectively tolerises regulatory M2 Mφs with little effect on pro-inflammatory M1 Mφs; differential suppression facilitating immunopathology at the expense of immunity.

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## 1. Introduction

Chronic periodontitis (CP) is a chronic relapsing-remitting inflammatory disease of periodontal tissues, cycling between bouts of inflammation and immune suppression, resulting in destruction of the periodontium and ultimately tooth loss. CP results from persistent microbial challenge by dysbiotic biofilms in which *Porphyromonas gingivalis* (PG) is an important member,

inducing host inflammatory responses (Ximenez-Fyvie, Haffajee, & Socransky, 2000; Ezzo & Cutler, 2003). PG is an intracellular oral pathogen, which infects mucosal macrophages (Mφs) (Cutler, Arnold, & Schenkein, 1993; Yilmaz, Watanabe, & Lamont, 2002). Clearance of which would normally require cell mediated immune responses, however PG-lipopolysaccharide (PG-LPS) induces immune-deviation towards non-clearing humoral responses, facilitating pathogen persistence (Pulendran et al., 2001). PG-LPS also exhibits low endotoxin activity and structural variation, hence differential utilisation of TLR2 and TLR4 (Darveau et al., 2004); thus, subverting both adaptive and innate immunity to survive in mucosal tissue. Immunosuppression can drive this immune deviation. PG-LPS suppresses Mφ responses by inducing endotoxin tolerance (ET); where LPS pre-exposure rendered innate immune cells refractory to subsequent endotoxin challenge (Biswas & Lopez-Collazo, 2009). ET may be beneficial or harmful

**Abbreviations:** Mφs, macrophages; IL-10, interleukin-10; IL-10R, interleukin-10 Receptor; TNFα, tumour necrosis factor-α; CP, chronic periodontitis; PG-LPS, porphyromonas gingivalis lipopolysaccharide; TLR, toll-like receptor; ET, endotoxin tolerance; STAT-3, signal transducer and activation of transcription-3; NFκB, nuclear factor-kappa B; SOCS-3, suppressor of cytokine signalling-3; PAMPs, pathogen associated molecular patterns.

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<http://dx.doi.org/10.1016/j.archoralbio.2016.10.029>

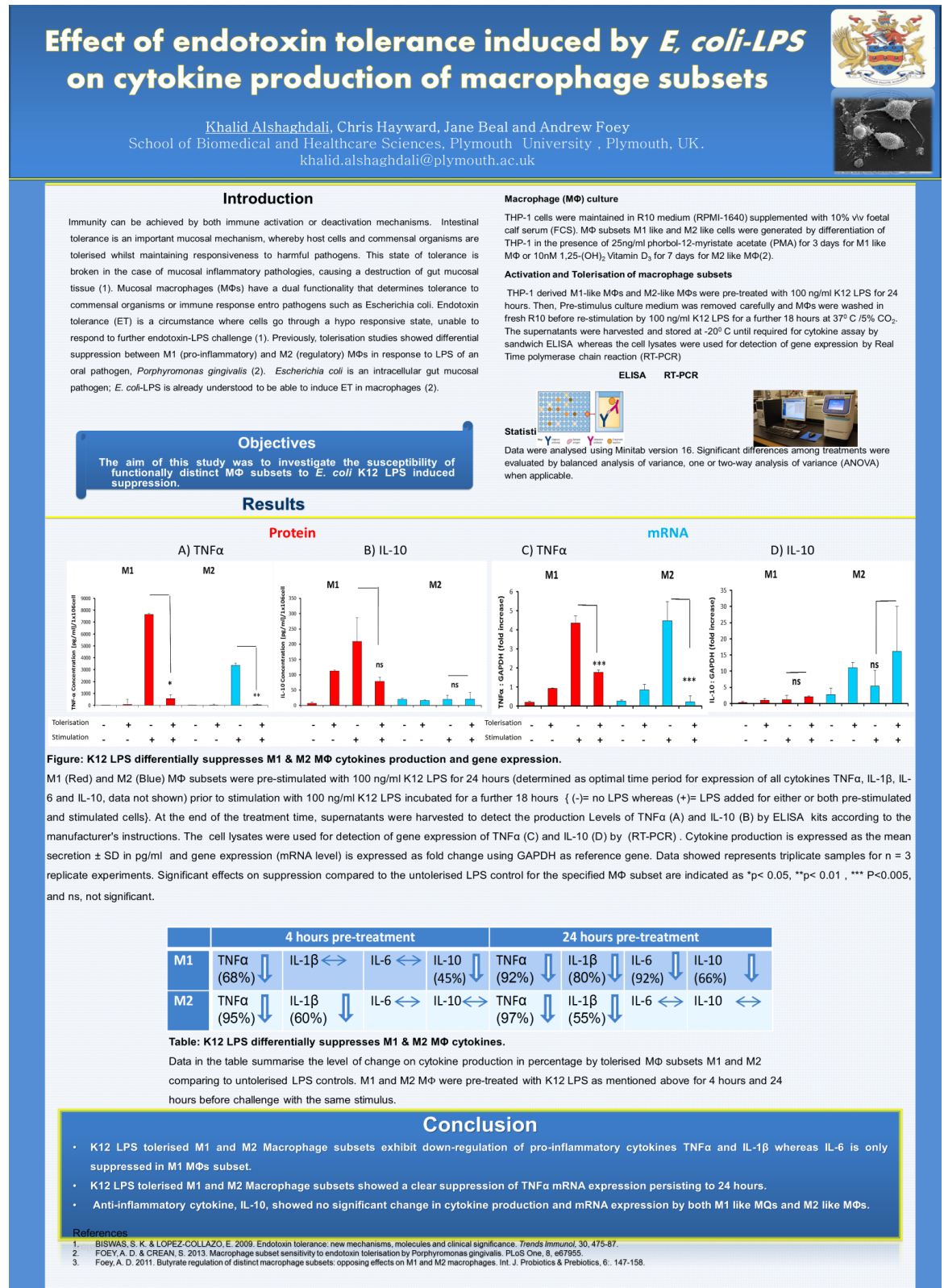
0003-9969/© 2016 Published by Elsevier Ltd.

**Macrophage subset sensitivity to endotoxin tolerance is determined by distinct expression profiles of pattern recognition receptors and negative regulators**


**To be published in mid of November**

## Poster Presentations

- 1) Al-shaghhdali, K., Hayward, C., Beal, J. and Foey, A. (2014): Effect of endotoxin tolerance induced by *E. coli*-LPS on cytokine production of macrophage subsets. British Society for Immunology Annual Congress, 1–4 December 2014, Brighton, UK.




- 2) Al-Shaghhdali, K., Hayward, C., Beal, J. and Foey, A. (2016): Negative regulatory molecules involved in endotoxin tolerance induced macrophage subsets. 9th Saudi Students' Conference, 13th-14th February, ICC Birmingham, UK.



**Negative regulatory molecules involved in endotoxin tolerance induced in Macrophage subsets**

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School of Biomedical and Healthcare Sciences, Plymouth University, Plymouth, UK.  
khalid.alshaghhdali@plymouth.ac.uk



9th Saudi Students' Conference - UK

## Introduction

Immune deactivation mechanisms are no less important than immune activation in the immune system. Intestinal tolerance is a vital mucosal mechanism, where host cells are tolerated whilst maintaining responsiveness to harmful pathogens. This state of tolerance is broken in the case of mucosal inflammatory pathologies, causing a destruction of gut mucosal tissue (1). Endotoxin tolerance (ET) is a circumstance where cells go through a hypo responsive state, unable to respond to further endotoxin-LPS challenge (1). As essential components of the innate immune system, macrophage cells (MΦs) can interact directly with pathogens as well as instruct the adaptive immune response. Mucosal MΦs have a dual functionality that determines tolerance to commensal organisms or immune response entropathogens such as *Escherichia coli*. Previous observations in our laboratory have demonstrated a differential subset-specific sensitivity to ET mediated by LPS from the oral pathogen, *Porphyromonas gingivalis* (PG) (2). *E. coli* is an intracellular gut mucosal pathogen which have an natural ability to induce ET in macrophages (2). ET induced by MΦs exists by different mechanisms such as down-regulation of PRRs (e.g.TLR4) or shedding of cytokine receptors and PRRs and/or induction of negative regulatory molecules, which have a role function in inhibition TLR4 signal transduction, such as Tollip, Myd88s, SARM, IRAK-M and SIGIRR (2).

## Objectives

The aim of this study was to investigate ET mechanisms associated with MΦ subsets responding to the entropathogen *E. coli* K12.

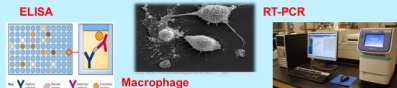
## Methods

### Macrophage (MΦ) culture

THP-1 cells were maintained in R10 medium (RPMI-1640) supplemented with 10% v/v foetal calf serum (FCS). MΦ subsets M1 like and M2 like cells were generated by differentiation of THP-1 in the presence of 25ng/ml phorbol-12-myristate acetate (PMA) for 3 days for M1 like MΦ or 10nM 1,25-(OH)<sub>2</sub> Vitamin D<sub>3</sub> for 7 days for M2 like MΦ(2).

### Activation and Tolerisation of macrophage subsets

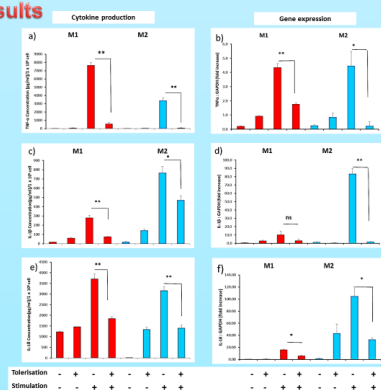
THP-1 derived M1-like MΦs and M2-like MΦs were pre-treated with 100 ng/ml K12 LPS for 24 hours. Then, Pre-stimulus culture medium was removed carefully and MΦs were washed in fresh R10 before re-stimulation by 100 ng/ml K12 LPS for a further 18 hours at 37° C/5% CO<sub>2</sub>. The supernatants were harvested and stored at -20° C until required for cytokine assay by sandwich ELISA whereas the cell lysates were used for detection of gene expression by Real Time polymerase chain reaction (RT-PCR).



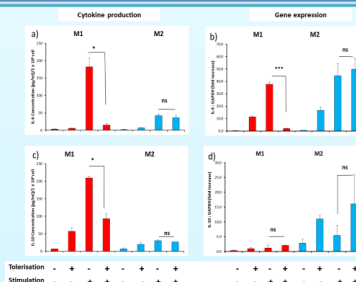
### Statistical analysis

Data were analysed using Minitab version 16. Significant differences among treatments were evaluated by balanced analysis of variance, one or two-way analysis of variance (ANOVA) when applicable.

## Results



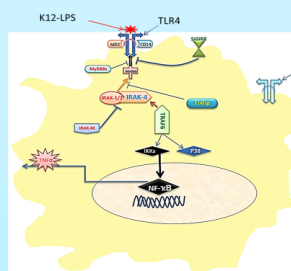
**Figure 1: The suppression of TNFα, IL-1β and IL-18, pro-inflammatory cytokines, in protein secretion and gene expression by *E.coli* K12-LPS tolerised M1 & M2 MΦs.** M1 (red) and M2 (Blue) MΦ subsets were pre-stimulated with 100 ng/ml K12 LPS for 24 hours prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18 hours (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. Cytokine production is expressed as the mean secretion  $\pm$  SD in pg/ml for TNFα (a), IL-1β (c), IL-18 (e). Gene expression (mRNA level) for TNFα(b), IL-1β(d), IL-18(f) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001[3]) using 2<sup>-ΔΔCT</sup>. Data showed represents triplicate samples for n = 3 replicate experiments. Significant effects on suppression compared to the unstimulated LPS control for the specified MΦ subset are indicated as \* p < 0.05, \*\* p < 0.01 and ns = no significant.



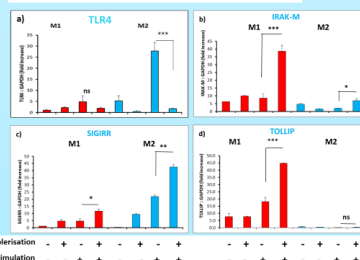
**Figure 2: *E.coli* K12-LPS differentially suppress IL-6 and IL-10, anti-inflammatory cytokines, in protein secretion and gene expression by tolerised M1 & M2 MΦs.**

**Table: M1 and M2 MΦs are differentially response to endotoxin tolerance induced by different PAMPs**

Stimulus	K12- LPS			PG- LPS		
Cytokine induced	TNFα	IL-1β	IL-6	TNFα	IL-1β	IL-6
M1-like	7656	281	183	1200	37	110
Control*	$\pm 362$	$\pm 28$	$\pm 25$	$\pm 300$	$\pm 30$	$\pm 4$
M1-like Tolerised	496	75	15	1700	50	105
	$\pm 162$	$\pm 4$	$\pm 4$	$\pm 30$	$\pm 5$	$\pm 10$
	93% ↓	73% ↓	92% ↓	41% ↓	35% ↓	5% ↓
M2-like control*	3366	765	42	800	90	110
	$\pm 309$	$\pm 70$	$\pm 5$	$\pm 150$	$\pm 80$	$\pm 30$
M2-like Tolerised	61	471	36	10	75	575
	$\pm 1$	$\pm 18$	$\pm 7$	$\pm 3$	$\pm 10$	$\pm 30$
	98% ↓	38% ↓	14% ↓	98% ↓	16% ↓	475% ↓



**Figure 3: Toll-like receptor 4 TLR4 pathway and its negative regulation in endotoxin tolerance.** *E.coli* Lipopolysaccharide (LPS (K12-LPS); TLR4, Toll-like receptor 4; TLR2, Toll-like receptor 2; CD14; MD2, myeloid differentiation protein-2; MyD88, myeloid differentiation primary response 88; MyD88s, myeloid differentiation primary response 88 short version; IRAK-1,2,4 & M, IL-1 receptor-associated kinase; Tollip, Toll interacting protein; TRAF6, TNF receptor-associated factor-6; SIGIRR, Single Immunoglobulin IL-1 Receptor Related molecule; IKKs, IκB kinases; NF-κB, nuclear factor kappa B; P38, P38 mitogen-activated protein kinases. As adapted from Cavallion and Adib-Conquy, 2006 (4).



**Figure 4: The involvement of TLR4 receptor and negative regulators in endotoxin-tolerance mechanisms in MΦ subsets.**

## Conclusion


- ET induced by *E. coli* K12-LPS failed to demonstrate a differential subset-specific response.
- A significant decrease in the expression and secretion of pro-inflammatory cytokine production and no significant change in anti-inflammatory cytokine, IL-10.
- ET showed suppression of TLR4 expression whereas augmentation of IRAK-M in both re-stimulated tolerised MΦ subsets.
- Unlike PG- LPS, *E. coli* K12-LPS tolerises MΦs pro-inflammatory cytokine production which is associated with corresponding up-regulation of IL-10 and the negative regulators e.g. (IRAK-M), in a subset-independent manner.

## References

- BISWAS, S. K. & LOPEZ-COLLADO, E. 2009. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends Immunol.* 30, 475-87.
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- 3) Al-Shaghhdali, K., Hayward, C., Beal, J. and Foey, A. (2016): Endotoxin tolerance mechanisms in macrophage subsets tolerised by *E. coli* LPS. PUPSMD Annual Research Student Conference, 14 April 2016, St Mellion, Cornwall.





## Endotoxin tolerance mechanisms in macrophage subsets tolerised by *E. coli* LPS

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جامعة هائل  
University of Hail

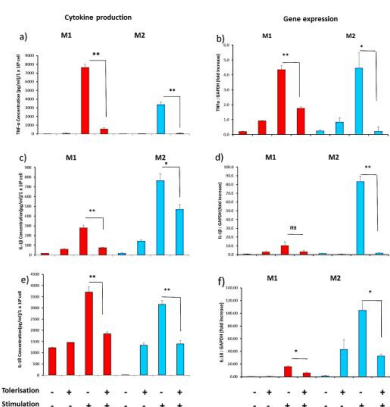
### Introduction

Immune deactivation mechanisms are no less important than immune activation in the immune system. Intestinal tolerance is a vital mucosal mechanism, where host cells are tolerised whilst maintaining responsiveness to harmful pathogens. This state of tolerance is broken in the case of mucosal inflammatory pathologies, causing a destruction of gut mucosal tissue (1). Endotoxin tolerance (ET) is a condition where cells go through a hypo responsive state, incapable to respond to extra endotoxin-LPS challenge (1). As essential components of the innate immune system, macrophage cells (MΦs) can interact directly with pathogens as well as instruct the adaptive immune response. Mucosal MΦs have a dual functionality that determines tolerance to commensal organisms or immune response to pathogens such as *Escherichia coli*. *E. coli* is an intracellular gut mucosal pathogen which has a natural ability to induce ET in macrophages (2). ET induced by MΦs exists by different mechanisms such as the regulation of PRRs include TLR4, TLR2 and NOD2 or shedding of cytokine receptors and PRRs. Negative regulatory molecules such as IRAK-M, Tollip, SIGIRR, SOCS3 and STAT3 have a role function in ET intercellular mechanisms (2).

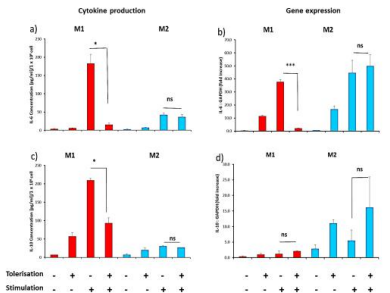
### Objectives

The aim of this study was to investigate ET mechanisms related with MΦ subsets responding to endotoxin challenge by the enteropathogen *E. coli* K12.

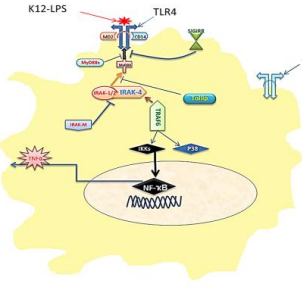
### Results



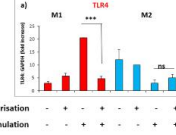
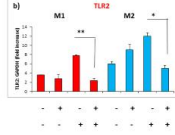
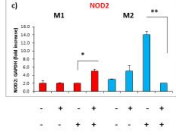
**Figure 1: The suppression of TNFα, IL-1β and IL-18, pro-inflammatory cytokines, in protein secretion and gene expression by *E. coli* K12-LPS tolerised M1 & M2 MΦs.** M1 (red) and M2 (Blue) MΦ subsets were pre-stimulated with 100 ng/ml K12 LPS for 24 hours prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18 hours (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. Cytokine production is expressed as the mean secretion ± SD in pg/ml for TNFα (a), IL-1β (c), IL-18 (e). IL-18 (f) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al., 2001)(3) using 2<sup>-ΔΔCt</sup>. Data showed represents triplicate samples for n = 3 replicate experiments. Significant effects on suppression compared to the unstimulated LPS control for the specified MΦ subset are indicated as \* p < 0.05, \*\* p < 0.01 and ns = no significant.



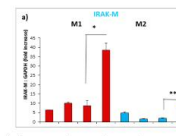
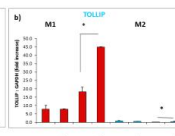
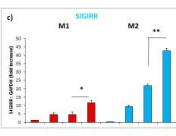
**Figure 2: *E. coli* K12-LPS differentially suppress IL-6 and IL-10, anti-inflammatory cytokines, in protein secretion and gene expression by tolerised M1 & M2 MΦs.**

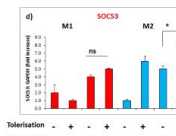
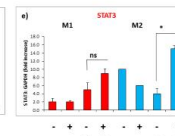


**Figure 3: Toll-like receptor 4 (TLR4) pathway and its negative regulation in endotoxin tolerance.** *E. coli* Lipopolysaccharide (LPS) (K12-LPS); TLR4, Toll-like receptor 4; TLR2, Toll-like receptor 2; CD14, MyD88, myeloid differentiation protein-2; MyD88, myeloid differentiation primary response 88; MyD88s, myeloid differentiation primary response 88 short version; IRAK-1,2,4 & M, IL-1 receptor-associated kinase; Tollip, Toll interacting protein; TRAF6, TNF receptor-associated factor-6; SIGIRR, Single Immunoglobulin IL-1 Receptor Related molecule; IKKs, IκB kinases; NF-κB, nuclear factor kappa B; P38, P38 mitogen-activated protein kinases. As adapted from Cavillon and Adib-Conquy, 2006 (4).

**Figure 4: The involvement of TLR4 (a), TLR2 (b) and NOD2 (c) receptor in endotoxin-tolerance mechanisms in MΦ subsets.**

**Figure 5: The involvement of negative regulators in endotoxin-tolerance mechanisms in MΦ subsets. IRAK-M(a), TOLLIP (b), SIGIRR (c), SOCS3 (d) and STAT3 (e).**

### Conclusion

- ET induced by *E. coli* K12-LPS failed to demonstrate a differential subset-specific response.
- A significant decrease in the expression and secretion of pro-inflammatory cytokine production and no significant change in anti-inflammatory cytokine, IL-10.
- ET showed differential modulation of PRRs gene expression i.e. suppression of TLR4 and increase of NOD2 in M1 MΦs whereas no change in TLR4 and decrease in NOD2 in M2 MΦs that may related with the differential regulation of the negative regulators.
- E. coli* K12-LPS tolerises MΦs pro-inflammatory cytokine production which is associated with corresponding up-regulation of IL-10 and the negative regulators e.g. (IRAK-M), in a subset-independent manner.

### Methods

**Macrophage (MΦ) culture**

THP-1 cells were maintained in R10 medium (RPMI-1640) supplemented with 10% v/v foetal calf serum (FCS). MΦ subsets M1 like and M2 like cells were generated by differentiation of THP-1 in the presence of 25ng/ml phorbol-12-myristate acetate (PMA) for 3 days for M1 like MΦ or 10nM 1,25-(OH)<sub>2</sub> Vitamin D<sub>3</sub> for 7 days for M2 like MΦ(2).

**Activation and Toleration of macrophage subsets**

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**Statistical analysis**

Data were analysed using Minitab version 16. Significant differences among treatments were evaluated by balanced analysis of variance, one or two-way analysis of variance (ANOVA) when applicable.

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- BISWAS, S. K. & LOPEZ-COLLAZO, E. 2009. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends Immunol.* 30, 475-87.
- FOEY, A. D. & CREAN, S. 2013. Macrophage subset sensitivity to endotoxin toleration by *Porphyromonas gingivalis*. *PLoS One* 8, e67855.
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## Endotoxin tolerisation differentially modulates MΦ cytokine production and IRAK-M expression in a subset dependent manner

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Innate immunity  
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### Introduction

Immune deactivation mechanisms are no less important than immune activation in the immune system. Intestinal tolerance is a vital mucosal mechanism, where host cells are tolerised whilst maintaining responsiveness to harmful pathogens. This condition of tolerance is broken in the case of mucosal inflammatory pathologies, causing a destruction of gut mucosal tissue (1). Endotoxin tolerisation (ET) is a phenomenon where cells go through a hypo responsive state, unable to respond to further endotoxin-LPS challenge (1). As essential components of the innate immune system, macrophage cells (MΦs) can interact directly with pathogens as well as instruct the adaptive immune response. Mucosal MΦs have a dual functionality that determines tolerance to commensal organisms or immune response onto pathogens such as *Escherichia coli*. *E. coli* is an intracellular gut mucosal pathogen which have a natural ability to induce ET in macrophages (2). The key readout for ET in these cells was the strong reduction of TNFα production, pro-inflammatory cytokine, as compared to the cells exposed to endotoxin only once ET induced by MΦs exists by different mechanisms such as down-regulation of PRRs (e.g. TLR4) or shedding of cytokine receptors and PRRs and/or induction of negative regulatory molecules, which have a role function in inhibition TLR4 signal transduction, such as IRAK-M, Tollip, Myd88s, SARM, and SIGIRR (2).

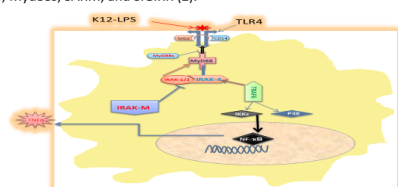


Figure 1: Toll-like receptor 4 TLR4 pathway and its negative regulation in endotoxin tolerance. *E. coli* Lipopolysaccharide LPS (K12-LPS), TLR4, Toll-like receptor 4; CD14, MD2, myeloid differentiation protein-2; MyD88, myeloid differentiation primary response 88; MyD88s, myeloid differentiation primary response 88 short version; IRAK-1,2,4 &M, IL-1 receptor-associated kinase; TRAF6, TNF receptor-associated factor-6; IKKs, IκB kinases; NF-κB, nuclear factor kappa B; P38, P38 mitogen-activated protein kinases. As adapted from Cavallion and Adib-Conquy, 2006 (3).

### Objectives

The aim of this study was to explore ET processes linked with MΦ subsets responding to the enteropathogen *E. coli* K12.

### Methods

#### Macrophage (MΦ) culture

THP-1 cells were maintained in R10 medium (RPMI-1640) supplemented with 10% v/v foetal calf serum (FCS). MΦ subsets M1-like and M2-like cells were generated by differentiation of THP-1 in the presence of 25ng/ml phorbol-12-myristate acetate (PMA) for 3 days for M1 like MΦ or 10nM 1,25-(OH)<sub>2</sub> Vitamin D<sub>3</sub> for 7 days for M2 like MΦ(2).

#### Activation and Tolerisation of macrophage subsets

THP-1 derived M1-like MΦs and M2-like MΦs were pre-treated with 100 ng/ml K12 LPS for 24 hours. Then, Pre-stimulus culture medium was removed carefully and MΦs were washed in fresh R10 before re-stimulation by 100 ng/ml K12 LPS for a further 18 hours at 37° C /5% CO<sub>2</sub>. The supernatants were harvested and stored at -20° C until required for cytokine assay by sandwich ELISA and gene expression by Real Time polymerase chain reaction (RT-PCR). The cell lysates were used for detection of IRAK-M protein level by Western Blot and the cell extract were used to read out gene expression by (RT-PCR).

#### Statistical analysis

Data were analysed using Minitab version 16. Significant differences among treatments were evaluated by balanced analysis of variance, one or two-way analysis of variance (ANOVA) when applicable.

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### Results

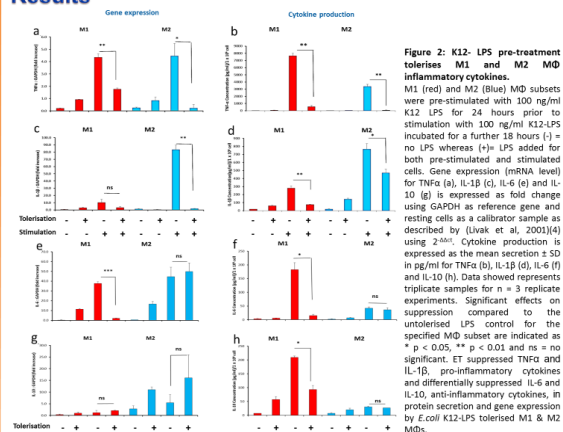


Figure 2: K12- LPS pre-treatment tolerises M1 and M2 MΦ inflammatory cytokines. M1 (red) and M2 (Blue) MΦ subsets were pre-stimulated with 100 ng/ml K12 LPS for 24 hours prior to stimulation with 100 ng/ml K12-LPS incubated for a further 18 hours (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells. Gene expression (mRNA level) for TNFα (a), IL-1β (c), IL-6 (e) and IL-10 (g) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al., 2001)(4) using 2<sup>-ΔΔCT</sup>. Cytokine production is expressed as the mean secretion ± SD in pg/ml for TNFα (b), IL-1β (d), IL-6 (f) and IL-10 (h). Data showed represents triplicate samples for n = 3 replicate experiments. Significant effects on suppression compared to the unstimulated LPS control for the specified MΦ subset are indicated as \* p < 0.05, \*\* p < 0.01 and ns = no significant. ET suppressed TNFα and IL-1β, pro-inflammatory cytokines and differentially suppressed IL-6 and IL-10, anti-inflammatory cytokines. In protein secretion and gene expression by *E. coli* K12-LPS tolerised M1 & M2 MΦs.

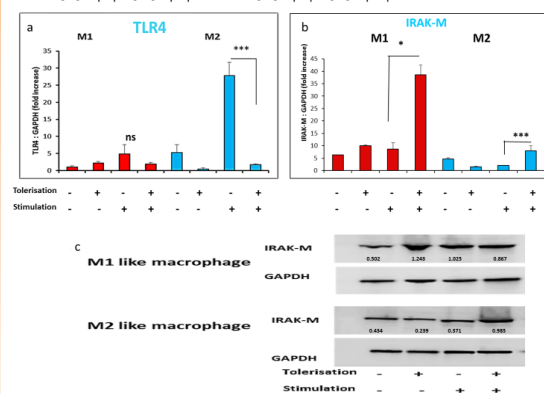


Figure 3: Endotoxin tolerisation modulates expression of MΦ TLR4 and negative regulatory IRAK-M. Gene expression (mRNA level) for TLR4 (a) and IRAK-M (b) are expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al., 2001)(4) using 2<sup>-ΔΔCT</sup>. Data showed represents triplicate samples for n = 3 replicate experiments. Significant effects on suppression compared to the unstimulated LPS control for the specified MΦ subset are indicated as \* p < 0.05, \*\* p < 0.01 and ns = no significant. Western blot for IRAK-M level (c) in MΦ subsets. WB analysis shows augmentation in IRAK-M level for M2 MΦs. Loading controls are presented as GAPDH plots below the corresponding IRAK-M-Westerns. Data displayed are representative of three replicate experiments.

### Conclusion

- Tolerised M1- and M2-like MΦs exhibited a significant reduction in expression and secretion of pro-inflammatory cytokines and comparable levels of anti-inflammatory cytokine, IL-10, compared to unstimulated cells, where M2 MΦs showed rise in IL-10 production and M1 MΦs did not.
- Tolerised M2-like MΦs showed more significant suppression of TLR4 expression.
- ET showed an augmentation of IRAK-M gene expression in both re-stimulated tolerised MΦ subsets, whereas only M2-like MΦs presented up-regulation in IRAK-M protein levels.
- E. coli* LPS tolerises pro-inflammatory cytokine production in MΦ subsets which differentially relies on various mechanisms, such as the effect of anti-inflammatory IL-10 and the expression of IRAK-M in M2 MΦs.

## **Membership:**

British society for Immunology (BSI) membership no 24851

## **Postgraduate Skills Training:**

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